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# Studies Directed Toward the Isolation and Structure Determination of Sex and Food Attractant of *Cylas Formicarius Elegantulus* (Sum.).

Angelo Russo

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STUDIES DIRECTED TOWARD THE ISOLATION AND STRUCTURE  
DETERMINATION OF SEX AND FOOD ATTRACTANT OF  
CYLAS FORMICARIUS ELEGANTULUS (SUM.)

A Dissertation

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of

Doctor of Philosophy

in

The Department of Biochemistry

by

Angelo Russo  
B.S., Louisiana State University, 1969

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To Sisyphus

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## ABSTRACT

This study is an attempt to isolate and determine the structure of the sex and food attractant of the sweet potato weevil (Cylas formicarius elegantulus). Adsorbent chromatographic techniques on Silicar and Alumina were initially probed to facilitate the separation of the pheromone from inactive material. These techniques did not lend themselves to the isolation of the sex attractant. Biological response of the weevils could not be observed after a biologically active fraction had been treated with either one of the adsorbent surfaces. Whole-body extractions of mixed sex weevils likewise were not an effective means of isolating the sex attractant. Here there was no biologically active material. However, sublimation of the fecal extract did provide a successful medium for initial separation of the pheromone.

In research on the food attractant, olfactometry did not offer an appropriate means of biologically monitoring an isolation scheme. The olfactometers results did demonstrate the importance of humidity or moisture on biologically testing. Multiple attempts



were then made to discover a suitable surface (vegetables, papers, agar) for bioassaying. Corks best answered this need. When corks were treated with extracts from the lyophilized skin of the sweet potato, it was found that there was a quantitative response, based on the amount of extract applied, the size of the cork, and the environment of the weevils. In a pseudo-natural environment, it appears that as the size of the cork or the amount of the extract decreases, so also does the average biting response. Industrial sources of potato skin, both alkali and steam treated, were biologically assayed in the hope of obtaining copious source materials. The alkali treated potato was inactive, while the steam treated potato was only slightly active.

Sublimation was used in an attempted isolation of the food attractant from the sweet potato. The sublimed material was subjected to gas chromatography/mass spectral analysis. Nine methyl esters (methyl tetradecanoate, methyl pentadecanoate, methyl hexadecanoate, methyl heptadecanoate, methyl octadecanoate, methyl octadecdienoate, methyl octadectrienoate, methyl docosanoate, methyl eicosanoate), two straight chained hydrocarbons (n-heneicosane, n-tricosane), and

squalene were identified and confirmed as constituents of the sublimate. None of these showed any biological activity. However, on one gas chromatographic/mass spectral analysis, 2,6-di-t-butyl-4-methylphenol and phenol were identified. Of these two compounds, phenol elicited a feeding response. Thirteen phenolic esters (phenyl acetate, phenyl propionate, phenyl butanoate, phenyl pentanoate, phenyl hexanoate, phenyl heptanoate, phenyl octanoate, phenyl nonanoate, phenyl decanoate, phenyl dodecanoate, phenyl tetradecanoate, phenyl hexadecanoate and phenyl octadecanoate) were then synthesized and tested for biological activity. There was a small amount of activity associated with phenyl heptanoate, phenyl octanoate, and phenyl nonanoate, and a positive gradient level of response from the phenyl butanoate to phenol itself.

## CHAPTER I

### HISTORY

Scientists have continually sought new methods to control pests since the plagues of biblical times. Yet, it was not until the early part of the twentieth century that serious experimentation was performed to discover the natural processes involved in the life cycle of malevolent insects with the hope that such discoveries would suggest ways of exterminating them effectively. The intensity of this experimentation, however, was attenuated with the introduction of non-host specific insecticides providing a means of adequately controlling insidious pests. Unfortunately, the indiscriminate use of these insecticides was scientifically myopic because their non-specificity created an ecological crisis. Scientists have once again focused their attention on ways of controlling these insects without resorting to universally deleterious chemicals. The annals of their research attest to the many problems which have beset them in the essential task of interfering with the communication systems of insects, thereby foiling their ability to

reproduce themselves. Two of the most promising means of interfering with the communication systems of insects are found in research on sex and food attractants.

Since the beginning of man's history, he has sought means to rid himself of annoying pests by redirecting them through food concoctions with obvious attracting power. In the first century A.D., for instance, Pliny the Elder in the Historia Naturalis recommended that a dead fish be suspended next to a tree to attract the ants which would normally decimate the foliage of that tree.<sup>1</sup> Likewise, the earliest shepherds concerned themselves with the destruction of the insects preying on their flocks. They, however, turned more to repellents than to attractants, because these seemed the most feasible and direct method of attack. The value of the attractant at that time was not so obvious. In fact, it was not until 1885 that an attractant was used for economic purposes. In that year Coquillett attempted to control grasshoppers by using poisoned bait to attract them.<sup>2</sup> Subsequently, European wine growers constructed traps of stale beer, brown sugar, and rum to thwart the moths which ravished their grapevines. Still later, the addition of oranges and

lemons increased the efficiency of grasshopper baits.<sup>3</sup> It was in 1922, however, that a chemical basis for scientific experimentation on food attractants emerged when Parker substituted amyl alcohol for the citrus fruit in grasshopper baits.<sup>4</sup>

Even though there was rudimentary experimentation in these early years, scientists were still seriously disputing both the center and precise function of olfaction in insects. Nebulous experimentation and questionable results are so voluminous and disparate, that it is difficult to determine a scientific progression in the midst of such confusion. For instance, men like Burmeister (1836)<sup>5</sup> and Dumeril (1823)<sup>6</sup> theorized that the olfactory organ was posited either in the stigmata or the trachae. Ramdohr (1811)<sup>7</sup>, on the other hand, confused the olfaction seat with salivary glands in the head. Rosenthal (1811)<sup>8</sup> and Huber (1814)<sup>9</sup> in their turn, respectively located this organ in the chitinous folds between the two antennae and the buccal cavity of the honey bee. However, it was not until the observations made by Fabre<sup>10</sup> in 1900 that a basis for accretive understanding of sex attractants was set.

At the turn of the twentieth century, Fabre discovered the cocoon containing an oak moth which to that time

had eluded his search. He placed this cocoon in a netted cage on his desk near a window. A female moth subsequently emerged and to his astonishment, that night up to sixty male oak moths, rare to that area, were hovering near the window. It was at this point that he began experiments designed to understand what caused the male moths to congregate around the netted cage. He placed the female moth in an air-tight, transparent container and noted that the males were no longer attracted to her, but rather to the cage from which she had recently been removed. He then returned the female moth to the original cage, observing that while from afar the male moths were directly attracted to the cage, proximately, they experienced a confused state of behavior. They lighted in the immediate vicinity of the cage and subsequently groped for the exact position of the female. Additionally, Fabre noted that the presence of chemical odors from hydrogen sulfide, naphthalene, and tobacco smoke did not interfere with the ability of the male moth to find the female. He conducted similar experiments on the female emperor moth with the same results. Then, excising the antennae of the male moth, he correctly observed that it was this organ which enabled the male to locate the female.

Similar experiments were carried out by Mayer<sup>11</sup> and Soule on promethea and gypsy moths. A wooden box was rigged containing virgin females. Air was blown over the females and exited through an aperture into a chimney. The male moths were always attracted to the top of the chimney, regardless of interference by chemicals such as carbon disulfide or diethyl sulfide; thereby he showed that the eyes had no bearing on the male's ability to find the female. In another experiment, he determined that male moths were attracted to severed abdominal sections from virgin females.

Despite the fact that evidence was compounding to demonstrate that most of the insects were perceiving their mates by behavioral responses to something impinging on the antennae, McIndoo,<sup>12</sup> nevertheless, argued that this evidence was scientifically invalid because the insects involved in the experimentation were tested under abnormal circumstances. According to him, pasting or excising the antennae constituted a mutilation of natural processes. He stated that the antennae were not the olfactory site. However, concurrently, von Frisch<sup>13</sup> by delicately removing the antennae from bees, concluded that such excisions did not effect the entire normal systemic function of these

insects, but indeed did interfere with their ability to perceive odor. Subsequently, Minnich,<sup>14</sup> a student of von Frisch, determined that while the antennae were the primary site of olfaction, other parts of the insect body were involved in the process.

As early as 1913, theories were being advanced about the very nature of olfaction. Teudt,<sup>15</sup> for instance, advocated that molecules impinging on nerve endings transmit their internal vibrations. Amoore,<sup>16</sup> on the other hand, suggested an olfaction theory based on the stereochemical nature of the molecule as it fits into the receptor site. He proposed that certain stereochemical configurations impart primary odors, such as musky and floral; secondary odors arise from a complex combination of primary odors. Callahan<sup>17</sup> argued that attraction is based on infrared radiation between insects in flight, while Wright<sup>18</sup> contended that attractancy is the result of a low frequency radiation from the attractant molecules measured in the Raman region which is delineated by an unknown site on the antennal surface. Associated with this site is a pigment molecule matching the frequency of the attractant. These modern theories, however, are not the result of blind chance. The fundamental experimentation on attractants in the late



nineteenth and early twentieth centuries significantly contributed to the current thinking on both food and sex attractants. Richards,<sup>19</sup> for instance, in an impressive review of the work done on scent glands in insects, demonstrates the monumental tasks that confronted the pioneers in this type of endeavor.

In the late 1920's, Richards stated that the female exudant of the scent gland functions to bring the two sexes together; whereas the scent gland in the male usually stimulates the female to copulate. Relying on the scent gland as a sexual attractant, entomologists felt that it might be possible to draw insects to sites for trapping and killing. For instance, Forbush<sup>20</sup> and Fernald tried to control the gypsy moth by using virgin female baited traps. His efforts were futile because not enough moths were destroyed to affect the population density; however, the traps did offer a method for detecting and monitoring infestation sites. Efforts to refine this method eventually led to the conclusion that the abdominal sections closest to the pudenda were just as effective in attracting the insects as was the live bait.<sup>21</sup> Subsequently, extracts of these abdominal tips were used as the lure. It is important to note that these extracts were highly efficient when

employed as substitutes for the virgin female.

Scientists had now progressed to the point where they were ready to study the extracts with the thought of isolating the pure chemical or chemicals responsible for these biological phenomena.

A biogenetic division of contemporaneous experimentation on these chemicals seems felicitous in that such a categorization may ultimately offer a clearer scientific perspective of sex attractants. The biogenetic division has been subsectioned into three groups: acetogenins, terpenoids, and a combination of aromatics and alkaloids. Table 1 indicates the sex attractants that are acetogenins.

Even though the stage was set for isolating sex attractants in their pure form as early as 1930, it was not until 1957 that the first report of such an isolation and structure determination was made. Amin<sup>22</sup> stated that dimethylamine was the chemical which was responsible for sex attraction in Bombyx mori. However, Butenandt<sup>23</sup> and Hecker in the following year pointed out that although dimethylamine did elicit a behavioral response in Bombyx mori at a concentration level of 10 micrograms per milliliter of solvent, the true sex attractant stimulates activity at a concentration level as low as  $10^{-5}$  micrograms per milliliter. Even octatrienol,

Table 1. Acetogenins

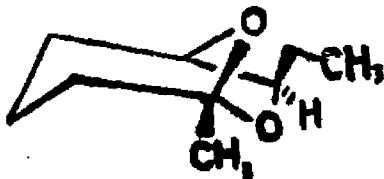
Structure	Insect Species
$\text{HO-CH}_2\text{-(CH}_2)_8\text{-CH}^{\text{E}}\text{=CH-CH}^{\text{Z}}\text{=CH-(CH}_2)_2\text{-CH}_3$	Silkworm ( <u>Bombyx mori</u> ) <sup>27</sup>
$\text{CH}_3\text{CH}_3\text{CH-(CH}_2)_4\text{-CH}^{\text{O}}\text{-CH-(CH}_2)_9\text{-CH}_3$	Gypsy moth ( <u>Porthetria dispar</u> ) <sup>36,37</sup>
$\text{CH}_3\text{CO}_2\text{CH}_2\text{-(CH}_2)_5\text{OCH}^{\text{Z}}\text{=CH-(CH}_2)_3\text{-CH}_3$	Cabbage looper moth ( <u>Trichoplusia ni</u> ) <sup>50,51</sup>
$\text{CH}_3\text{CO}_2\text{CH}_2\text{-(CH}_2)_7\text{-CH}^{\text{Z}}\text{=CH-(CH}_2)_3\text{-CH}_3$ (+) $\text{CH}_3\text{CO}_2\text{CH}_2\text{-(CH}_2)_9\text{-CH}^{\text{Z}}\text{=CH-CH}_2\text{-CH}_3$	Tortricid moths ( <u>Adoxophyes orana</u> , <u>Clepsis spectrana</u> ) <sup>54,55</sup>
	Western pine beetle ( <u>Dendroctonus brevicornis</u> ) <sup>61</sup>

Table 1 (Continued)

Structure	Insect Species
$\text{CH}_3-(\text{CH}_2)_3-\text{CO}_2\text{H}$	Sugarbeet wireworm ( <u>Limonius californicus</u> ) <sup>62</sup>
$\text{HOCH}_2-(\text{CH}_2)_6-\text{CH}^{\text{E}}=\text{CH}-\text{CH}^{\text{E}}=\text{CH}-\text{CH}_3$	Codling moth ( <u>Laspeyresia pomonella</u> ) <sup>65</sup>
$\text{CH}_3\text{CO}_2\text{CH}_2-(\text{CH}_2)_9-\text{CH}^{\text{E}}=\text{CH}-\text{CH}_2-\text{CH}_3$	Larch bud moth ( <u>Zeiraphera diniana</u> ) <sup>66</sup>
$\text{C}_8\text{H}_{17}-\text{CH}^{\text{Z}}=\text{CH}-\text{C}_{13}\text{H}_{27}$	House fly ( <u>Musca domestica</u> ) <sup>67</sup>
$\text{CH}_3\text{CH}_3\text{CH}-\text{CH}_2-\text{CO}_2\text{CH}_2-(\text{CH}_2)_3-\text{CH}^{\text{Z}}=\text{CH}-(\text{CH}_2)_2-\text{CH}_3$	Pine emperor moth ( <u>Nudaurelia cytherea cytherea</u> ) <sup>116</sup>
$\text{CH}_3\text{CO}_2\text{CH}_2-(\text{CH}_2)_6-\text{CH}^{\text{Z}}=\text{CH}-(\text{CH}_2)_2-\text{CH}_3$	Oriental fruit moth ( <u>Grapholitha molesta</u> ) <sup>117</sup>
$\text{CH}_3\text{CO}_2\text{CH}_2-(\text{CH}_2)_7-\text{CH}^{\text{Z}}=\text{CH}-\text{CH}_2-\text{CH}_3$	Grapeberry moth ( <u>Paralobesia viteana</u> ) <sup>118</sup>

Table 1 (Continued)

Structure	Insect Species
$\text{CH}_3\text{CO}_2\text{CH}_2(\text{CH}_2)_9\text{-CH}^{\text{Z}}\text{=CH-CH}_2\text{CH}_3$	Redbanded leafroller ( <u>Argyrotaenia velutinana</u> ) <sup>119</sup> (+) Obliquebanded leafroller ( <u>Choristoneura rosaceana</u> ) <sup>120</sup> (+) European corn borer ( <u>Ostrinia nubilalis</u> , Iowa) <sup>121</sup>
$\text{HOCH}_2\text{-CH}^{\text{Z}}\text{=C}^{\text{CH}_2\text{-CH}_3}\text{-(CH}_2\text{)}_2\text{-CH}^{\text{E}}\text{=CH-(CH}_2\text{)}_2\text{-CH}_3$	Codling moth ( <u>Laspeyresia pomonella</u> ) <sup>122</sup>
$\text{CH}_3\text{CO}_2\text{CH}_2\text{-(CH}_2\text{)-CH}_3$	False codling moth ( <u>Argyroplece leucotreta</u> ) <sup>123</sup>

Table 1 (Continued)

Structure	Insect Species
	<u>Rachiplusia ou</u> <sup>53</sup>
	(+)
	<u>Pseudoplusia includens</u> <sup>53</sup>
	(+)
	<u>Autographa biloba</u> <sup>53</sup>
	(+)
	<u>Autographa californica</u> <sup>52</sup>
$\text{CH}_3\text{CO}_2\text{CH}_2-(\text{CH}_2)_4-\overset{\text{Z}}{\text{CH}}=\text{CH}-(\text{CH}_2)_3-\text{CH}_3$	
$\text{CH}_3\text{CO}_2\text{CH}_2-(\text{CH}_2)_7-\overset{\text{Z}}{\text{CH}}=\text{CH}-(\text{CH}_2)_3-\text{CH}_3$	Fall armyworm moth ( <u>Spodoptera frugiperda</u> ) <sup>124</sup>

Table 1 (Continued)

Structure	Insect Species
$\text{CH}_3\text{CO}_2\text{CH}_2-(\text{CH}_2)_7-\overset{\text{Z}}{\text{CH}}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_3$	Mediterranean flour moth ( <u>Anagasta kuehniella</u> ) <sup>125</sup> (+) Beet armyworm moth ( <u>Spodoptera exigua</u> ) <sup>126</sup>
$\text{HOOC}-\overset{\text{Z}}{\text{CH}}=\text{CH}-(\text{CH}_2)_5-\text{COCH}_3$ (+) $\text{HOOC}-\overset{\text{Z}}{\text{CH}}=\text{CH}-(\text{CH}_2)_5-\text{CHOHCH}_3$	Honey bee ( <u>Apis mellifera</u> ) <sup>127</sup>
$\text{HOOC}-\text{CH}_2-\overset{\text{E}}{\text{CH}}=\text{CH}-\overset{\text{Z}}{\text{CH}}=\text{CH}-(\text{CH}_2)_7-\text{CH}_3$	Black carpet beetle ( <u>Attagenus megatoma</u> ) <sup>128</sup>

Table 1 (Continued)

Structure	Insect Species
$\text{CH}_3\text{OOC}-(\text{CH}_2)_6-\overset{\text{Z}}{\text{CH}=\text{CH}}-(\text{CH}_2)_4-\text{CHCH}_3-\text{CH}_2\text{CH}_3$ <p style="text-align: center;">(+)</p> $\text{HOCH}_2-(\text{CH}_2)_6-\overset{\text{Z}}{\text{CH}=\text{CH}}-(\text{CH}_2)_4-\text{CHCH}_3-\text{CH}_2\text{CH}_3$	Dermestid beetle ( <u>Trogoderma</u> <u>inclusum</u> ) <sup>129</sup>
$\text{CH}_3\text{OOC}-\overset{\text{E}}{\text{CH}=\text{CH}}-\text{CH}=\text{C}=\text{CH}-(\text{CH}_2)_7-\text{CH}_3$	Bean weevil ( <u>Acanthoscelides</u> <u>obtectus</u> ) <sup>130</sup>
$\text{CH}_3\text{CO}_2\text{CH}_2-(\text{CH}_2)_7-\overset{\text{Z}}{\text{CH}=\text{CH}}-\text{CH}_2-\overset{\text{E}}{\text{CH}=\text{CH}}-\text{CH}_3$	Indian meal moth ( <u>Plodia</u> <u>interpunctella</u> ) <sup>131</sup> Almond moth ( <u>Cadra</u> <u>cautella</u> ) <sup>131</sup>



Table 1 (Continued)

Structure	Insect Species
$\text{CH}_3\text{CO}_2\text{CH}_2-(\text{CH}_2)_7-\overset{\text{Z}}{\text{CH}=\text{CH}}-\text{CH}_2-\overset{\text{E}}{\text{CH}=\text{CH}}\text{CH}_3$ <p>(+)</p> $\text{CH}_3\text{CO}_2\text{CH}_2-(\text{CH}_2)_7-\text{CH}=\text{CH}-(\text{CH}_2)_3-\text{CH}_3$	Southern armyworm moth ( <u>Prodenia</u> <u>eridania</u> ) <sup>132</sup>

according to Butenandt and Hecker, which produces a biological response at a concentration level of 1 microgram per milliliter, is 1000 times more active than Amin's reported compound; yet, octatrienol is 100,000 times less potent than the natural sex attractant. Butenandt's rebuttal of Amin's findings emphasizes the problems that beset chemical scientists in their attempts to isolate sex attractants in a pure form. Initially there was no conception of the minute quantity of this substance actually present in the extracts.

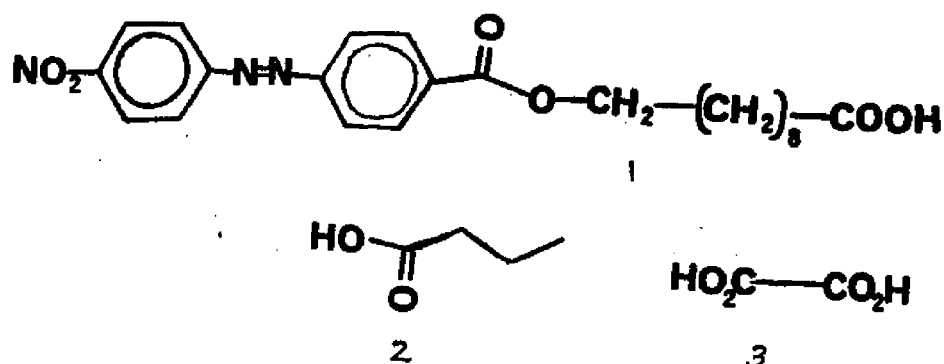
In 1959,<sup>24</sup> Butenandt actually isolated the first natural sex attractant from Bombyx mori. Concomitantly, he reviewed the whole field of sex attraction and provided conceptual clarification by innovating a restrictive nomenclature. It was he who first employed the word pheromone: "The word is derived from the Green pherein (to carry) and horman (to excite, to stimulate). 'Pheromone' should designate substances that are secreted by an animal to the outside and cause a specific reaction in a receiving individual of the same species, e.g., a release of a certain behavior or a determination of physiologic development."<sup>25</sup> Butenandt points out that pheromones act as chemical messengers, and consequently presuppose a social function among insects.

The bioassay used by Butenandt in the progress of isolating the pure sex attractant involved the observation of the behavioral response of the Bombyx mori males when they were exposed to samples containing the sex attractant. The response was easily monitored visually because the male moths when confronted with the sex attractant became "generally excited, beating their wings, and dancing erratically; at high concentration they attempt to copulate with the odiferous object."<sup>25</sup> Substances for assaying were diluted with petroleum ether. A glass rod was wetted, removed, and brought to within one-half of one centimeter of the antennae of the male moths. All the concentration levels that Butenandt tested were bioassayed on twenty moths. These twenty insects enabled him to construct a quasi-quantification method whereby he could evaluate his enrichment procedure. When a minimum of ten insects responded positively to the bioassayed substance, Butenandt arbitrarily termed this particular concentration level a "sex attractant unit." Consequently, he was able to distinguish differences in concentration levels of 1:10 with some certainty. After establishing a method whereby a physiologically potent sex attractant in a minute

quantity could be monitored, Butenandt et al. proceeded with their isolation.<sup>26</sup> They collected 313,000 scent glands from virgin female moths; these were stored in and extracted with petroleum ether. The extract was hydrolyzed with alkali, and the neutral fraction was shown to be biologically active and, investigated. From this neutral fraction, the alcoholic compounds were further separated by transforming them into their respective succinic mono-esters. These mono-esters were then hydrolyzed and transformed to their p-nitroazobenzenecarboxylic esters, thereby preventing loss of active material because the non-esterified alcohols were volatile. Neither ester showed physiological activity; however, when the esters were hydrolyzed, physiological activity was again apparent. The esterified alcohols were purified by chromatography on Alumina in conjunction with repeated Craig distributions. Finally, paper and distribution chromatography yielded a pure compound. The compound was then hydrolyzed; it showed one "sex attractant unit" activity at  $10^{-5}$  micrograms per milliliter. The separation was arduous and the yield, extremely low. From 500,000 scent glands only 12 milligrams of the p-nitroazobenzenecarboxylic ester, NABS ester, of the sex attractant were isolated.

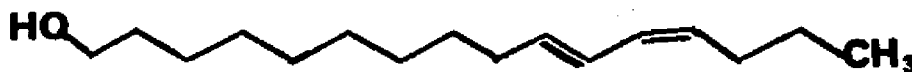
Even with this biologically active material on hand, the structure of the sex attractant was still not available. In order to determine the correct structure, Butenandt et al.<sup>27</sup> used information gleaned from elemental analysis of the NABS ester which was shown to be  $C_{29}H_{37}N_3O_4$ . This elemental analysis implied that the molecular formula of the underivatized pheromone was  $C_{16}H_{30}O$ . In addition to the molecular formula, which suggests the presence of two double bonds, ultra-violet absorbance at 230 millimicrons plus infrared absorbance at 10.18 and 10.56 microns reinforced the concept of two sites of unsaturation and pointed to a cis-trans configuration. Catalytic hydrogenation of the pheromone resulted in the isolation of a chemical having a melting point at 47-48°C. Cetyl alcohol proved to have the same melting point; additionally, a mixed melting point showed no depression. In order to substantiate the  $C_{16}$  backbone, Butenandt et al. made the -naphthyl urethane derivative of both cetyl alcohol and the hydrogenated sex attractant. These derivatives also were shown to have the same melting point and mixed melting point. Following oxidation of the NABS ester of the sex attractant by  $KMnO_4$  in dry acetone, he isolated the NABS- $\omega$ -hydroxydecanoic acid 1, butanoic acid 2, and oxalic acid 3 (Figure 1).

Figure 1



By means of these fragments, Butenandt et al. deduced the structure of bombykol as the acetogenin hexadecadien-1-ol, possessing a 10-cis, 12-trans geometry. After the synthetic cis-trans isomer was shown to have biological activity at a level of only  $10^{-2}$  micrograms, the validity of the entire structure was questioned. It was not until 1961,<sup>28</sup> after the synthesis of all four possible geometric isomers of the diene, that the correct structure of the sex attractant was proved to be 10-trans, 12-cis, hexadecadien-1-ol (Figure 2).

Figure 2



The pure, synthetic bombykol, obtained independently by two groups,<sup>29,30</sup> showed biological potency greater than that elicited from the natural sex attractant. The synthetic bombykol evoked a biological response at  $10^{-13}$  micrograms; the natural sex attractant, at  $10^{-10}$  micrograms.

Along with the isolation and structuring of the natural sex attractant from Bombyx mori, ancillary data illustrated the complexity of isolation and structure determination of sex attractants. For example, Butenandt et al.<sup>31</sup> demonstrated that a number of alcoholic compounds displayed a quasi-gradient level of attraction as the structural similarity to the true pheromone was approached. This observation cautioned scientists that they should not confuse these quasi-gradient levels of attraction with the ultimate sex attractant itself.

These ancillary data also suggested a means by which the presence of biogenetic precursors of the pheromone could be utilized. Butenandt et al. took inactive, crude mixtures of materials isolated from the female moth, reduced them with lithium aluminum hydride, and produced biologically active material. This technique worked to some extent; however, the materials that were isolated, although possessing biological activity, were far less potent than bombykol.

Scheider,<sup>32</sup> in collaboration with Butenandt, additionally developed a new method for probing the effects of biologically active material. This technique, described by Schneider, consisted in monitoring the change of electric potential in the antennae when exposed to various chemical stimuli. Ultimately, it required that a single, micro, silver chloride coated electrode be placed into a recently excised insect antenna.<sup>33</sup> The exposure of this antenna to a chemical could be monitored by the action potential as displayed on an oscilloscope. It was observed that quasi-pheromones only in high concentration could evoke an electrochemical response.<sup>24</sup> The pure pheromone, however, could elicit an intense response even in miniscule amounts, a response which was reproducible and subject

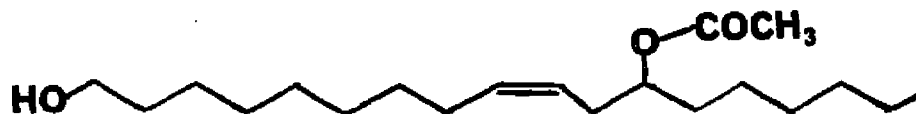


to concentration changes. This electroantennographic technique offered a sensitive and ostensibly precise means of following his purification scheme.

Scientists of the United States Department of Agriculture, working independently of Butenandt's group, arrived at the same basic technique of isolating sex attractants. Their work on the gypsy moth pheromone, extending over a thirty year period, culminated with the publication in 1960 by Jacobson et al.<sup>34</sup> describing the isolation, structure determination, and synthesis of the acetogeninic natural sex attractant. This publication represented the first report on the veritable structure of an insect sex attractant. (The actual structure determination of bombykol was not accomplished until 1962.) In a manner not unlike that of Butenandt's, the Jacobson group effected the isolation of the sex attractant by first extracting 500,000 abdominal tips obtained from virgin female gypsy moths. The extract was subsequently saponified by means of boiling ethanolic alkali. The neutral fraction was then dissolved in acetone and cooled to  $-5^{\circ}\text{C}$ . The precipitate was filtered and tested for biological activity. It showed none. Consequently, the acetone soluble material became the subject of focus. Evaporation of the acetone yielded 75 milligrams

of a pale yellow viscous oil, containing the sex attractant. Its presence was demonstrated by biological assays on male moths both in the laboratory and in the field. The oil was then separated by paper chromatography, and only one spot had biological activity. Through this method of separation, Jacobson et al. was able to isolate 3.4 milligrams of a white waxy crystal and 20 milligrams of a colorless, blue fluorescent, petroleum ether soluble liquid. The petroleum ether soluble material showed biological activity at a concentration of  $10^{-7}$  micrograms; the white waxy material was only 25% as active. Gas chromatography of the liquid indicated that it was pure. Analysis of this sex attractant suggested that the molecular formula was either  $C_{17}H_{32}O_3$  or  $C_{18}H_{34}O_3$ . By virtue of infrared spectroscopy, hydrogenation, potassium permanganate and periodic acid oxidation, and optical rotation, the pheromone was identified as (+)-10-acetoxy-cis-7-hexadecen-1-ol (Figure 3).

Figure 3



The D&L form of Figure 3 was synthesized in 0.2% overall yield. Jacobson<sup>35</sup> successfully resolved the D&L racemate of the sex attractant as the brucine salts. The d enantiomer was identical in all respects to the natural sex attractant. He also demonstrated that the d,l mixture was biologically compared to the d enantiomer of the sex attractant showed no depressed level of activity.

Ten years after Jacobson et al. published the structure of the gypsy moth pheromone, he recognized that this structure was incorrect. The activity which they had observed was in reality caused by the presence of an impurity of the compound that he had isolated.<sup>36,37</sup> Bierl et al.<sup>38,39</sup> later isolated, identified, and synthesized the correct substance which is responsible for sex attractancy. From 78,000 abdominal tips, they acquired

enough material to run micro tests on thin layer chromatography. They observed that the active compound was probably a  $C_{18}$ - $C_{20}$  epoxide, and not the alcoholic ester that Jacobson had advanced. Synthesis of the sex attractant showed it to be cis-7,8-epoxy-2-methyl-octadecane. (Figure 4).

Figure 4

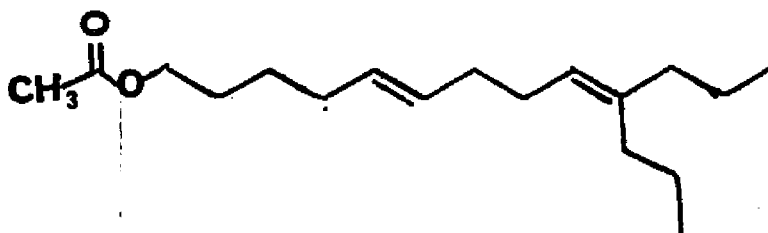


In field tests extending over a ten-day period, using ten female equivalents, 110 moths were caught by the synthetic sex lure; only three were trapped by the natural products.

After having erroneously reported the structure of the gypsy moth pheromone, Jones et al.<sup>40</sup> again proposed a biogenetically questionable structure for the pink bollworm moth pheromone. Nine years earlier,<sup>41</sup> it had been determined that such a sex attractant existed; in

1962, Ouye and Butt<sup>42</sup> supplied the additional information that extracts from the virgin female moth were an effective lure. Following these leads, Jones et al.<sup>40</sup> mascerated 850,000 virgin female moths, subjected the ostensible pheromone to classical structure determination, and arrived at the acetogenic compound, 10-propyl,trans-5,9-tridecadien-1-ol acetate commonly called "propylure."

Figure 5



This structure was corroborated by synthesis. The synthesized material appeared to be identical in all respects to the natural sex attractant. A number of more efficient syntheses have since been reported.<sup>43,44</sup>

Although effective in the laboratory, propylure was inactive in field tests. The crude extract, as Ouye<sup>42</sup> demonstrated, was capable of attracting the

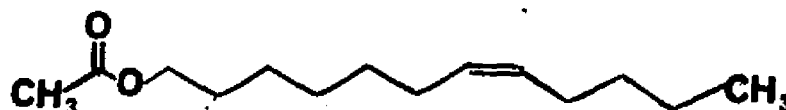
male moth in the field; however, when the pheromone was removed, neither the pheromone alone, nor the residue provoked activity. For the first time, there was evidence that a multiple phase pheromonal system existed.<sup>45</sup> Field activity was eventually elicited from propylure when N,N diethyl m-toluamide, isolated from the methanol insoluble extract of virgin females, was added to it. However, the activity was not nearly so impressive as that provoked by the crude extract.

Nonetheless, Eiter et al.<sup>43</sup> reported that propylure prepared by their group showed no biological activity whatsoever, thereby suggesting that the structure proposed by Jacobson on the pink bollworm pheromone was incorrect. Once again, as in the case of the American cockroach,<sup>46,47</sup> the credulity of Jacobson's work was questioned. Bearing the onus of proof, Jacobson<sup>48</sup> demonstrated that his structure was indeed valid; the failure that Eiter et al. experienced resulted from the fact that in synthesizing propylure, they also generated its geometric isomer--a masking compound. Jacobson showed that the propylure synthesized by Eiter's method was contaminated by 40% of the cis isomer. He pointed out that as little as 10% of the cis isomer as a contaminant strongly depressed the

response of the male moth; 15% totally extinguished it. Male moths exposed to the trans isomer within fifteen minutes after having been exposed to the cis isomer lost their sexual drive. Later, however, it was conceded that cis-7-hexadecen-1-ol acetate (hexalure), a more biogenetically reasonable structure, was significantly more potent than propylure.<sup>49</sup>

A pheromonal compound<sup>50,51</sup> analogous to hexalure was isolated from the cabbage looper moth and identified as cis-7-dodecen-1-ol acetate. (Figure 6).

Figure 6



The final structural proof was accomplished by synthesis of the sex attractant.

Of interest was Shorey's<sup>52</sup> observation that the extracts of female cabbage looper moths also excited

the male Autographa californica. Later, Berger and Canerday<sup>53</sup> demonstrated that the pheromone of the cabbage looper was effective in attracting males of Autographa biloba, Rachiplusia ou, and Pseudoplusia includens. In the extracts from the females of each of these three species, they noted the presence of a compound which had the same retention time as that of the cabbage looper. This was the first of many complex biological systems which would eventually arise. The subsequent experiments of Mink et al.,<sup>54</sup> for instance, again point out the complexity of research in pheromonal chemistry.

Minks et al.<sup>54</sup> determined the unique means by which Adoxophyes orana and Clepsia spectrana coexist in nature, with overlapping daily active periods and common acetogeninic sex attractants.<sup>55</sup> These tortricid moths maintain reproductive species isolation. Using varying admixtures of their common sex attractants, Minks et al. showed that a complex ecological interplay exists between the two species. A 9:1 concentration of cis-9-tetradecenyl acetate to cis-11-tetradecenyl acetate was maximally attractive to A. orana to the exclusion of C. spectrana; whereas C. spectrana was maximally attracted by a 4:1 ratio



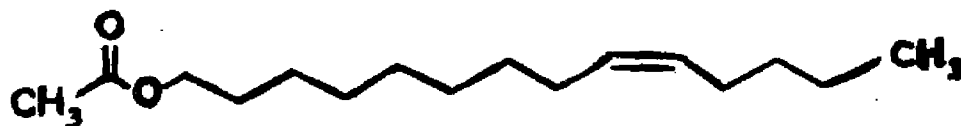
of cis-11 to cis-9 isomers. An even more complex biological system was reported by Riddiford and Williams.<sup>56,57</sup>

Riddiford and Williams demonstrated that some heat stable and water soluble chemical stimulates the virgin female polyphemous moth to release yet another chemical which in turn stimulates the male moth to find and copulate with the female. In terms of Butenandt's definition of pheromones,<sup>24</sup> the chemical-- a constituent of oak leaves, the chief food source of the moth--should not be considered an insect sex attractant; however, since the chemical is so intimately associated with pheromonal production, its categorization is difficult. The chemical, trans-2-hexenal, is in fact released into the air and must impinge upon the antennae of the female before she will begin producing the sex attractant.<sup>58</sup> Riddiford and Williams<sup>56</sup> suggest that the sensory input from the antennae to the brain must subsequently undergo some neuroendocrine relay. It was shown that Chanel No. 5 masked this relay system. Unlike oak leaves, other food sources of polyphemous, such as maple, birch, chestnut, elm, hickory, and beech leaves, did not stimulate the female to release her sex attractant.

Once finding herself in the vicinity of the correct host-plant, the female automatically starts to emit her sex lure. After she has mated, she is near a ready food source in which to oviposit. A biological advantage is gained through this intricate arrangement of lepidopteran host-plant-insect chemical signaling. Another example of the complex chemical signaling in lepidopterans is discussed in a taxonomic and evolutionary study by Roelofs and Comeau.<sup>59</sup>

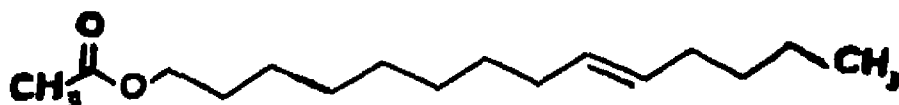
Roelofs and Comeau demonstrated strong pheromonal specificity between two species of tortricids (Bryotopha similis B1 and Bryotopha similis B2) which are identical with the exception of coloration: B1 is grayish, while B2 appears yellowish. When extracts of abdominal segments from either species were placed in the field, only the respective species of males were lured to the trap. In a search for the pheromone of the redbanded leafroller moth, they found B1 males responding to the acetogenin cis-9-tetradecen-1-ol acetate (Figure 7).

Figure 7



B2 males, on the other hand, were attracted by the trans-9-tetradecen-1-ol acetate. (Figure 8).

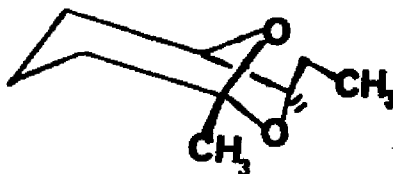
Figure 8



They also observed that if the two acetogeninic geometrical isomers were mixed, neither B1 nor B2 males were attracted. Roelofs and Comeau suggest that either of the geometric isomers acts as an inhibitor of sexual stimulation if perceived by the wrong Bryotopha male. This particular phenomenon represents mutational evolution of the enzyme systems of one species, thereby allowing chemical affinity for the active site, but no intrinsic activity. Inter-species copulation is thereby thwarted. The possibility that the pheromones of specific lepidopterans evolved from a common phylogenetic, archetypal template is not discounted by Roelofs.<sup>60</sup>

The work by Riddiford and Williams (vide page 31) on the intricate arrangement of the lepidopteran host-plant-insect chemical signaling is conceptually parallel to the experiments which Silverstein and coworkers performed. From the western pine beetle, Silverstein et al.<sup>61</sup> in 1968 isolated and structured the principal sex attractant. As usual, the isolation technique necessitated the mass rearing of insects so that sufficient frass could be collected. He extracted 1.6 kilograms of frass with benzene. Through column chromatography of the crude extract and subsequent vapor phase chromatographic separation, approximately 2 milligrams of pure active material were obtained. Data gathered from standard structure elucidation techniques allowed him to postulate the structure to be the exo-7-ethyl-5-methyl-6,8-dioxabicyclo 3.2.1 octane, commonly called "brevicomín" (Figure 9).

Figure 9



The structure was confirmed by stereospecific synthesis. The endo-isomer was also shown to be present in the frass. The biological activity of brevicomin synergistically increased when an inactive hydrocarbon mixture was added to it. One compound of this hydrocarbon mixture was identified as the monoterpene myrcene. The slightly active endo-isomer became highly active when myrcene was introduced. It should be pointed out that myrcene is released from the oleoresins of the host. When the advanced guard beetles are drawn to the western pine, their brevicomin coupled with myrcene signals the mass attack to commence. At the same time, brevicomin also lures Temnochil virescens chlorodia, the natural predator of the western pine beetle, to aggregate on trees under attack.

In order to arrive at the structure of brevicomin, 1.6 kilograms of frass were needed. This required the mass-rearing of insects. By contrast, Jacobson et al.<sup>62</sup> were able to isolate the pheromone of the sugarbeet wireworm<sup>63,64</sup> by using less than 100 insects. From only 18 virgin females, he collected 2.4 milligrams of an active fraction. The fraction was chromatographed and subsequently distilled. This process yielded valeric

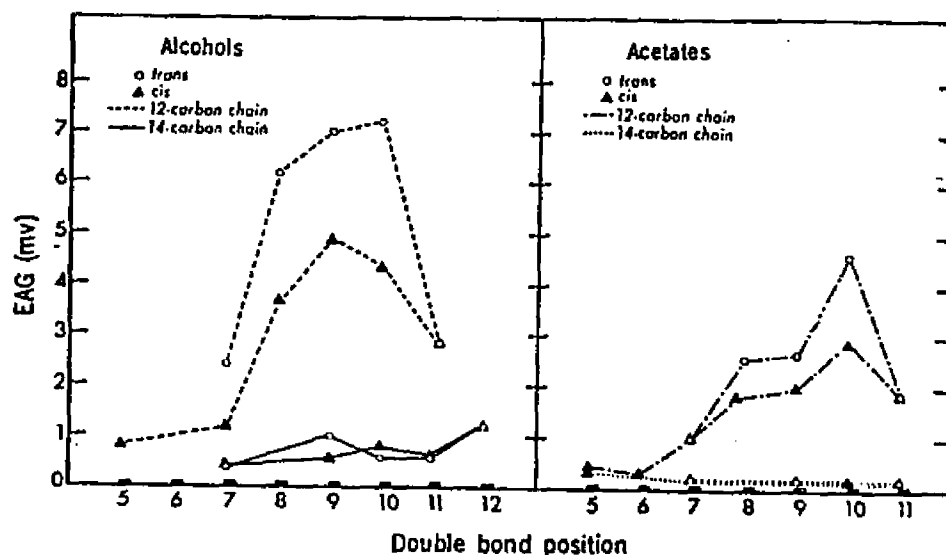
acid which was converted into its methyl ester and shown to be gas chromatographically pure. The beetles were attracted to a concentrated spot of valeric acid; however, as they came within close range, they showed signs of confusion and antennal irritation. Upon dilution of this spot, the antennal irritation ceased and the beetles were drawn directly to the sex attractant. It was hypothesized that the female of the sugarbeet wireworm stored large amounts (greater than 100 micrograms per female) of the pheromone and released it gradually.

Although Jacobson's requirement of a small quantity of insects was serendipitous, Roelofs et al.<sup>65,66</sup> did develop a novel and highly effective means of structuring sex attractants without the need of insect mass-rearing. Using electroantennographic techniques, they identified the structure of the acetogeninic pheromones in the codling and the larch bud moths, respectively. Their work was presaged by the experimentation of Schneider<sup>32,33</sup> and Butenandt<sup>24</sup>.

Working with only 50 female abdominal tips of the codling moth, they<sup>65</sup> ascertained the structure of the sex attractant by the use of gas chromatography

juxtaposed with electroantennography. By monitoring the insect response, they were able to focus their attention on the probable molecular functionality and chain length through gas chromatography retention time correlations. After this approximation, they used synthetic test chemicals in conjunction with the electroantennograms. These test chemicals showed the following gradient level (Figure 10):

Figure 10



The highest electroantennographic response indicated the structure to be trans-8-trans-10-dodec dien-1-ol (Figure 11).

Figure 11



Bioassays in the field indicated that one microliter of the postulated pheromone was capable of luring moths for a period longer than one month.

In a similar fashion, using only 200 abdominal tips, they determined the structure of the larch bud moth pheromone to be trans-11-tetradecenyl acetate<sup>66</sup> (Figure 12).

Figure 12





Field tests showed that when two microliters were placed in a polyethylene cap, attraction did not commence until forty days had passed. By optimizing the release rates, it was shown that 100 micrograms of pheromone were an overabundance; yet, after one month, traps baited with 100 micrograms became attractive. Subsequently, it was demonstrated that 10 micrograms and one nanogram quantities elicited the highest level of immediate attraction.

The first dipterous pheromone was found by Carlson et al.<sup>67</sup> The acetogeninic attractant is produced only by sexually mature female house flies which were surface washed with hexane or ethyl ether to remove cuticular lipids. Column chromatography of the crude wash yielded an active fraction with retention time similar to that of long-chained mono-olefins. Preparative gas chromatography of the active fraction allowed for the collection of most of the active peak corresponding to  $C_{23}$ . The position of the double bond was determined by micro-ozonolysis of a 10 microgram sample. The compound was shown to be cis-9-tricosene (Figure 13).

Figure 13



A 50 microgram sample of synthetic cis-isomer was more sexually potent than a 200 microgram sample of the trans-isomer or a 60 milligram sample of the crude fecal lipids.

While the majority of the sex attractants are acetogenins, a number of them are isoprenoid in origin (vide Table 2). The first terpenoid was shown to be present in the pheromonal system of a coleopteran. In 1966,<sup>68,69,70</sup> the beginning of a long series of papers by Silverstein's group on the subject of sex attractants in coleopterans appeared. Early work on Ips confusus by Pitman and Vite<sup>71</sup> and Wood<sup>72</sup> established that indeed a sex attractant existed, and that biological production of the pheromone was subject to the female's ingesting of its food. It was observed that the frass of the beetle, displaced from the bored hole as the insect ate, was biologically attractive.<sup>72</sup>

Table 2. Terpenoids

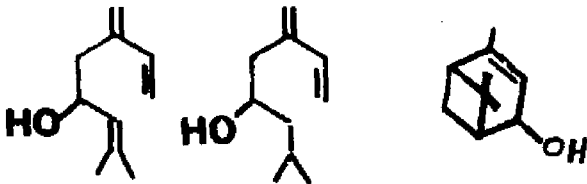
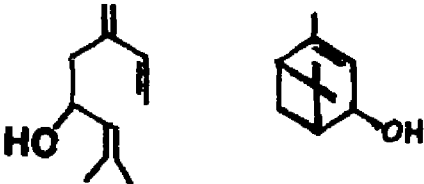
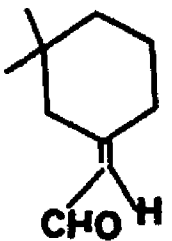
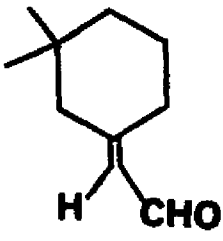
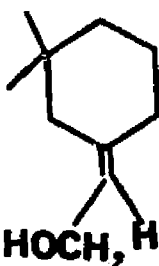
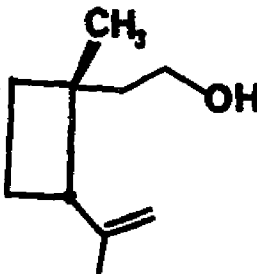
Structure	Insect Species
	<u>Ips confusus</u> <sup>68</sup>
	<u>Ips latidens</u> <sup>75</sup>

Table 2 (Continued)

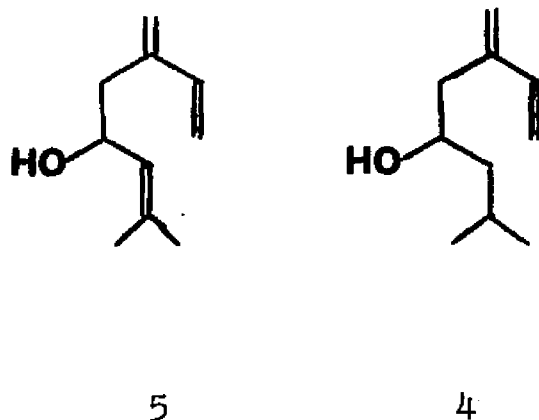
Structure	Insect Species
	<u>Dendroctonus frontalis</u> <sup>79</sup>
$\text{HO}-\text{CH}_2-(\text{CH}_2)_2-\text{CCH}_3^{\text{E}}=\text{CH}-(\text{CH}_2)_2-\text{CCH}_3^{\text{E}}=\text{CH}-\text{CH}_2-\text{OH}$	Danaid butterfly ( <u>Lycorea ceres</u> <u>ceres</u> ) <sup>82</sup>
$\text{HOOC}-(\text{CH}_2)_2-\text{CCH}_3^{\text{E}}=\text{CH}-(\text{CH}_2)_2-\text{CCH}_3^{\text{E}}=\text{CH}-\text{CH}_2-\text{OH}$	Monarch butterfly ( <u>Danaus</u> <u>plexippus</u> ) <sup>84</sup>

Table 2 (Continued)

Structure	Insect Species
	Boll weevil ( <u>Anthrenomus grandis</u> ) <sup>85</sup>
	
	

With this knowledge at hand, Silverstein et al.<sup>68</sup> proceeded to isolate from 3.0 kilograms of frass, two of the chemicals responsible for this activity. Their methodology consisted of a sequence of extractions, followed by short path vacuum distillation. The distillate was then subjected to column chromatography. At this point, only 0.0007% of the initial kilograms demonstrated any activity. Subsequently, the crude chromatographed material underwent further purification by means of vapor phase chromatography. It was noted that the retention time of the active fraction was bracketed by nonanal and geranyl acetate. Continued vapor phase chromatography gave a narrow cut, having sharp peaks at the leading and trailing ends. These two peaks were designated compounds 4 and 5 and became the focus of attention. By continuous use of gas chromatography, 260 milligrams of compound 4 and 26 milligrams of compound 5 were collected. After spectral analyses, compound 4 was shown to be the monoterpene (-)-2-methyl-6-methylene-7-octen-4-ol and compound 5, trans-2-methyl-6-methylene-3,7-octadien-2-ol. The second structure was later corrected to be the terpene 2-methyl-6-methylene-2,7-octadien-4-ol<sup>69</sup> (Figure 14).

Figure 14



There was no biological activity if only one compound was used as a sex attractant; however, this activity could be elicited if the two chemicals were mixed in microgram quantities. It became apparent that there was still another compound which made up the composite sex attractant. After repeating his procedure with an additional 1.5 kilogram of frass, the bicyclic monoterpene (+)-cis-verbenol (6) was identified as compound III.<sup>69</sup> All three compounds were synthesized,<sup>73</sup> thereby corroborating structural assignments. If one microgram of compound 4 was mixed with 0.01 microgram of compound 6, a biological response was evoked.<sup>74,75</sup> Despite this activity, assays both in the laboratory

and the field suggested that all of the pheromonal constituents in the frass were not accounted for. Nonetheless, Silverstein and his group<sup>69</sup> insisted that the first three compounds were in fact the principal sex attractants. Since the probability of multiple pheromonal composites is always present, they pointed out that no fraction in the workup can be discarded before it has been biologically assayed in combinations with other fractions.

The pheromonal complexity of coleopterans was also demonstrated in another set of experiments in which Ips latidens was attracted to a mixture of compounds 4 and 6 in both the laboratory and the field.<sup>75</sup> With the addition of compound 5, Ips latidens lost all affinity for the attractant. The synergistic and masking effect within the Ips beetles pose "some challenging chemical and biological problems."<sup>76</sup> Further tests<sup>75</sup> showed that two predators of the bark beetles are also attracted to mixtures of the ternary pheromonal system. It appears that the predators use the natural pheromones of the bark beetles as food attractants.

Silverstein had pointed out that the difficulties associated with isolating and structuring small

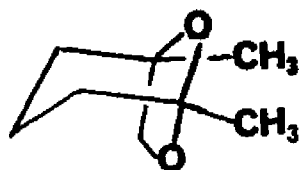


quantities of materials sometimes occasioned in temperature polemics.<sup>77</sup> Such polemics sprang from the work by Pitman et al.<sup>78</sup> concerning the role played by the bicyclic monoterpene trans-verbenol in attracting Dendroctonus. After they had isolated trans-verbenol from the hindgut of 5000 females, they stated that alone it had no activity; however, when mixed with other volatiles emitted from the host material of the beetles, it exhibited a synergistic effect. Additionally, when trans-verbenol was applied to infested billets, the attracting power of these billets was enhanced.<sup>79</sup> Such an enhancement was not observed when trans-verbenol was sprayed on non-infested billets. Vite and Pitman<sup>80</sup> subsequently proclaimed that there are two different mechanisms involved in the release of sex attractants by Dendroctonus and Ips. When Dendroctonus initially feeds on new host material, a high level of pheromone is released. This pheromone production, however, appears to be inhibited as feeding continues. They argued for the early presence in Dendroctonus of trans-verbenol which disappears within 12-16 hours after feeding has begun. They also postulated that trans-verbenol is responsible for pheromonal control in Dendroctonus. Ips, on the other hand,

continually released its pheromone throughout feeding. Silverstein's group<sup>81</sup> consistently questioned the role that trans-verbenol played in pheromonal production.

Kinzer et al.<sup>79</sup> reported another pheromone of the pine beetle. This compound was isolated from 6500 hind-guts of male beetles. Gas chromatographic separation, followed by spectral investigation, allowed them to propose tentatively the structure of the sex attractant to be 1,5-dimethyl-6,8-dioxabicyclo 3.2.1 octane. They names this compound "frontalin" (Figure 15).

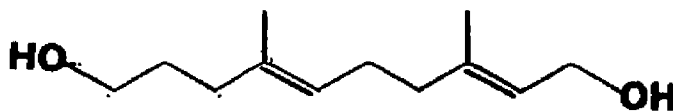
Figure 15



This structure was confirmed by synthesis. Frontalin showed very little biological activity alone; however, when trans-verbenol was added, a synergistic effect ensued. The mixture of frontalin and trans-verbenol was competitive with oleoresins added to 2500 crushed male southern pine beetles.

In his continuing study of danaiids, Meinwald et al.<sup>82</sup> published the structure of a chemical obtained from the queen butterfly. The males of this family possess a pair of extrusible appendages called "hair pencils." These hair pencils had long been suspected of being responsible for scent dissemination. They are extruded as the male pursues the female, and brushed against her antennae and head. The female, in turn, lights and copulation ensues. Meinwald et al.<sup>83</sup> had already isolated a heterocyclic aphrodisiac (vide infra) as a component of the active fraction. From the polar fraction, they separated the major component and by spectral data in conjunction with total synthesis, proved it to be the abridged oxidized sesquiterpene trans,trans-3,7-dimethyl-2,6-decadien-1,10 diol (Figure 16).

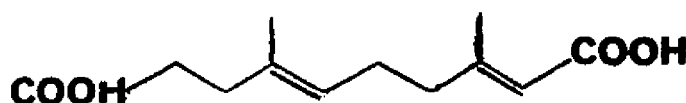
Figure 16



He observed that the biosynthesis of this compound is unknown; hence, it may be synthesized de novo or from an ingested food material which is a close metabolite of this aphrodisiac.

In a study similar to his work on the queen butterfly, Meinwald et al.<sup>84</sup> identified a component of the pheromonal secretion from the monarch butterfly. extracted 6000 hair pencils from male butterflies with dichloromethane, and by subsequent thin layer chromatography of the crude extract, isolated 11.8 milligrams of a colorless liquid. Again by using spectral data from infrared, nuclear magnetic resonance, and mass spectroscopy, they postulated another abridged oxidized sesquiterpene whose structure is trans,trans-3,7-dimethyl-2,6-decadien-1,10-dioic acid (Figure 17).

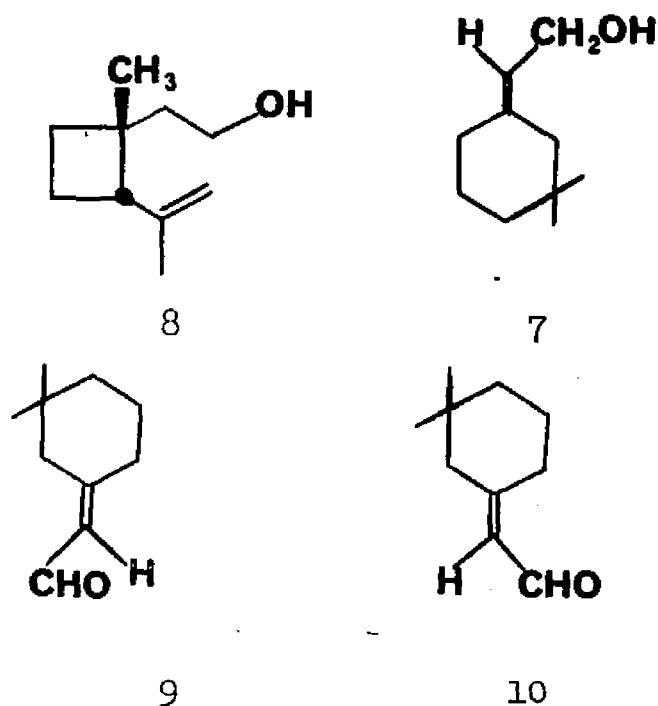
Figure 17



This postulated structure was confirmed by total synthesis.

Tumlinson et al.<sup>85</sup> in the same year reported the isolation, structural elucidation, and synthesis of the monoterpenoid boll weevil pheromones. Their monumental isolation was accomplished by extraction of 67,000 male boll weevils, 4.5 million mixed sex weevils, and 54.7 kilograms of frass, followed by steam distillation and subsequent multiple chromatographic separations. Through these separations they were able to isolate in milligram quantities, four active compounds. Alone, the four compounds elicited no biological activity; when mixed, they evoked maximum response from the female weevil. By means of spectral data and micro-ozonolysis, the structures of these four compounds were postulated to be cis-3,3-dimethyl-<sup>1</sup>, -cyclohexaneethanol (1), 2-isopropenyl-1-methyl-cyclobutaneethanol (8), trans-3,3-dimethyl-<sup>1</sup>, -cyclohexaneacetaldehyde (9), cis-3,3-dimethyl-<sup>1</sup>, -cyclohexaneacetaldehyde (10). All four structures were confirmed by synthesis (Figure 18).

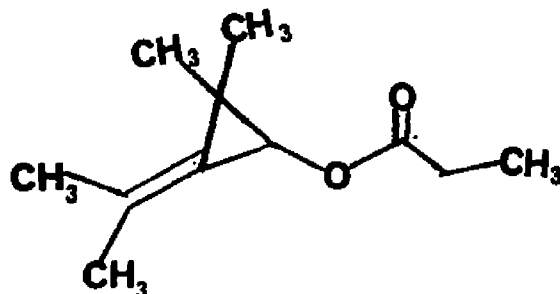
Figure 18



Jacobson and his group<sup>86</sup> in 1963 turned their attention to isolating and structuring what they believed to be the pheromone of the American cockroach. It had been shown in virgin female cockroaches, that the attractant adhered to filter paper on which the female had been placed.<sup>87</sup> Yamamoto, one of Jacobson's group,<sup>86</sup> devised a new method of obtaining the pheromone, not only of the American cockroach, but also of other insects as well. He placed virgin female cockroaches

in large metal containers and passed a stream of air over them. The vapors, mingling with the air, were trapped in dry-ice cooled flasks containing 4% HCl. The HCl was ostensibly required to stabilize the sex attractant. The condensate was removed and extracted with hexane; subsequently, the hexane layer was back extracted with water, then dried. After flash evaporation of the hexane at temperatures below 40°C, a residue was collected and chromatographed on a silicic acid column. By using this method of collection, the USDA group obtained 12.2 milligrams of pure attractant from 10,000 female cockroaches, milked continuously over a nine-month period. The pheromone elicited responses from males at concentrations as low as  $10^{-14}$  micrograms. It was shown to be pure by vapor phase chromatography. Elemental analysis suggested a  $C_{11}H_{18}O_2$  molecular formula. The compound exhibited no optical rotation, nor ultraviolet absorbence. Infrared spectroscopy revealed the presence of an ester. Following catalytic micro-reduction<sup>88</sup> and micro-oxidation,<sup>89</sup> Jacobson et al.<sup>86</sup> arrived at the biogenetically unlikely structure of 2,2-dimethyl-3-isopropylidene cyclopropyl propionate (Figure 19).

Figure 19



This structure biogenetically resembles the monoterpene chrysanthemic acid structure isolated from the family of compositae.

After synthesizing the supposed American cockroach pheromone which Jacobson had isolated and structured, Day and Whiting<sup>46</sup> proved that in reality this compound was not the true sex attractant. Subsequently, in a retraction, it was stated<sup>47</sup> that although the cockroach pheromone did not have the structure which had been advocated, it did have a molecular weight of 182 corresponding to  $C_{11}H_{18}O_2$ . This molecular weight was determined by double focus high resolution mass spectroscopy. Jacobson et al.<sup>47</sup> added that the attractant is chemically unstable, even at cold temperatures, and decomposes in a few days if kept



undiluted. However, if it is diluted with hydrocarbon solvents and refrigerated, it can be stored up to six months.

It is interesting to note that only one alkaloid and three aromatic compounds have been identified as components of the sexual communication system of insects (Table 3). The first of these was reported in 1968 by Alpin and Birch.<sup>90</sup> Their work involved three species of noctuid lepidopterans: Leucania impura, Leucania conigera, Phlogophora meticulosa. From the male brushes of these species, they isolated benzaldehyde, believing it not serendipitous, but rather aphrodisiac.

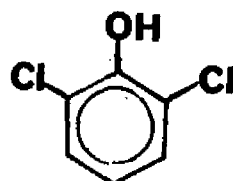
Henzell and Lowe<sup>91</sup> reported their isolation and structure determination of the second aromatic sex attractant which was found in the grass grub beetle. Extraction of 1500 virgin females yielded an active fraction which was short path vacuum distilled, and subsequently separated by thin-layer and gas chromatography. The substance was phenol. In the laboratory, male beetles responded to phenol at concentrations as low as 0.1 micrograms; in the field, they reacted to diluted phenol in concentrations as low as 100 parts per million. This pheromone is the first demonstration of a phenolic sex attractant.

Table 3. Aromatics and Alkaloids

Structure	Insect Species
Benzaldehyde	Noctuid moths ( <u>Leucania impura</u> , <u>Leucania conigera</u> , <u>Phlogophrora meticulosa</u> ) <sup>90</sup>
Phenol	Grass grub beetle ( <u>Costelytra</u> <u>zealandica</u> ) <sup>91</sup>
	Lone star tick ( <u>Amblyomma americanum</u> ) <sup>92</sup>
	Danaid butterfly ( <u>Lycorea ceres</u> <u>ceres</u> ) <sup>83</sup>

Berger<sup>92</sup> in 1972 published the structure of the pheromone of the lone star tick, the third aromatic pheromone. This sex attractant, like that of the grub beetle, was phenolic. By working with 400,000 females, he isolated two milligrams of pure sex attractant. After interpreting infrared spectra and mass spectrometry data, he arrived at the following biologically unlikely structure: 2,6-dichlorophenol.

Figure 20



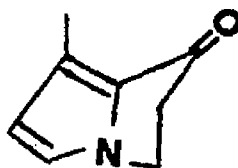
Synthetic 2,6-dichlorophenol was attractive at 0.1 micrograms when tested through gas chromatography eluates streaming over male ticks.

In an effort to ascertain if the natural pheromone was metabolically produced by the ticks or the result of selective collection from ingested

materials, Berger searched for the pheromone in the ticks' food source. None was present. He subsequently demonstrated that the sex attractant begins to appear in females four to five days after emergence; maximum concentrations are observed two weeks after emergence.

There has been only one report of an alkaloid that provokes a sexual biological response in insects. In 1966, Meinwald et al.<sup>83</sup> described the isolation and structure determination of insect aphrodisiacs obtained from danaid butterflies. In order to isolate the chemical responsible for the aphrodisiac phenomenon in the species Lycorea ceres ceres, they removed tufts of the hair pencils from 300 males and extracted them with either dichloromethane or carbon disulfide. Vapor phase chromatography of the extract showed the presence of three major components. Fractional sublimation of the extract residue afforded a crystalline compound with a melting point of 74-75°C. It smelled much like the natural secretion. By the use of infrared, ultraviolet, nuclear magnetic resonance, and mass spectroscopy, the structure of the crystalline solid was postulated to be 7-methyl-2, 3-dihydro-1H-pyrrolizidin-1-one (Figure 21).

Figure 21



The exact position of the methyl group was correlated by nuclear magnetic resonance and conclusively proved by synthesis.

Food attractants, unlike pheromones, do not readily lend themselves to categorization, primarily because there is a dearth of knowledge about the precise chemicals which lure the insects to their food sources; secondarily, because a biogenetic division, such as the one imposed upon the pheromones, would vitiate the continuity of much of the individual research in which there is interlinking experimentation on the acetogenins, terpenoids, etc. It therefore appears that the most germane method of presenting an historical review on food attractants is through chronological perspective.

The general tenor of early research in insect food attractants gained impetus in 1927 when Richmond<sup>93</sup> discovered that geraniol exerts exceptional attracting power on Japanese beetles. Shortly thereafter, Morgan and Lyon<sup>94</sup> demonstrated that amyl salicylate and amyl benzoate acted as feeding attractants for tobacco hornworm moths by inducing them to feed on artificial flowers.

Two years after, in 1929, Snapp and Swingle<sup>95</sup> showed that the plum curculio was slightly attracted by salicylaldehyde in the early part of the season and by gallic acid in the latter part of the season. Additionally, they demonstrated that the shothole borer was drawn to bark distillates and fermented water extracts of peach bark; the spotted cucumber beetle was attracted by oil of thyme and benzyl alcohol. Gnats of the family Chloropidae were especially enticed by isoamyl valerate.

In 1946, Langford and Cory<sup>96</sup> extended the work which Richmond had initiated in 1927 on the Japanese beetle. They found an alternative for the geraniol and eugenol as food attractants. This alternative consisted of an 8:2 admixture of caproic acid and

*p*-phenylethyl butyrate. If either of these chemicals was added to the normal bait, there was a great enhancement of activity.

Dethier<sup>97</sup> in 1952 demonstrated that isovaleraldehyde acted as a fly attractant. He experimented with isovaleraldehyde because he perceived its odor in the most alluring fly baits--10% malt extract or decayed casein. An interesting phenomenon associated with isovaleraldehyde was that it was an attractant at concentration levels of  $1.2 \times 10^{-5}M$ . Over ranges of  $2.0 \times 10^{-5}M$  to  $2.8 \times 10^{-5}M$ , isovaleraldehyde was shown to be partly repellent and partly attractive; at concentration levels greater than  $6.0 \times 10^{-5}M$ , isovaleraldehyde indicated maximum repellency. Dethier makes a salient comment on this seeming ambiguity:

. . . attractants and repellents are so intimately related that one could not be discussed and understood without inclusion of the other. One is the antithesis of the other yet a single substance may act as both. Two substances differing in concentration alone may elicit diametrically opposed reactions from an insect. It is this interrelationship which renders difficult a simple yet satisfactory definition in either case. Broadly speaking, an attractant is anything which draws; a repellent, anything which repels. More specifically, any stimulus which elicits a positive directive response may be termed an attractant; any stimulus which elicits an avoiding reaction may be termed a repellent.<sup>98</sup>

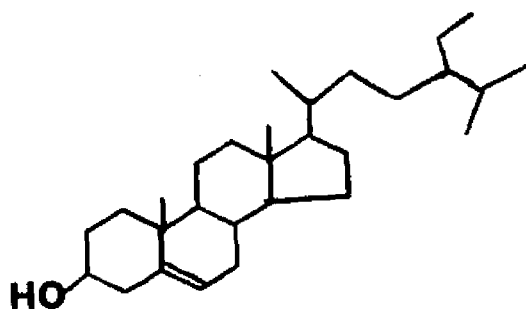
In 1958 Watanabe<sup>99</sup> isolated from steam distillates of mulberry leaves a number of compounds of which only  $\delta$ - $\gamma$ hexenol and  $\alpha$ - $\gamma$ hexenal showed attractancy characteristics for the silkworm larvae. Young larvae were more strongly drawn to  $\gamma$ - $\gamma$ hexenol; older larvae, to  $\alpha$ - $\gamma$ hexenal.

The work of Hamamura<sup>100</sup> on the feeding stimulus of Bombyx mori began in 1934<sup>101</sup> and lasted more than thirty years. The choice of the silkworm as a subject for investigation was auspicious because Bombyx mori is a good example of a monophagous insect, mulberry leaves being almost its exclusive diet. Hamamura's initial experiments were designed to isolate the food attractant from the volatile material exuded from the mulberry leaves. After methanol extraction of the mulberry leaves, the organic soluble material in addition to the residue was tested for biological activity. It was found that some substance within the methanol extract attracted larvae; however, the larvae showed no interest in feeding.<sup>102</sup> On the other hand, if the larvae were placed directly upon the residue, biting and feeding commenced. Hamamura<sup>102</sup> speculated that a complex biological program was operative wherein the separate existence of an



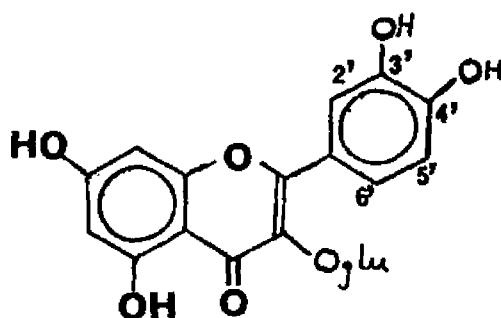
attracting, biting, and swallowing factor was requisite for feeding to be sustained. There were two biting factors present: one in the ether soluble material; the other in the methanol soluble material. He later demonstrated that -sitosterol (Figure 22) was the biting factor associated with the ether soluble material.<sup>103</sup>

Figure 22



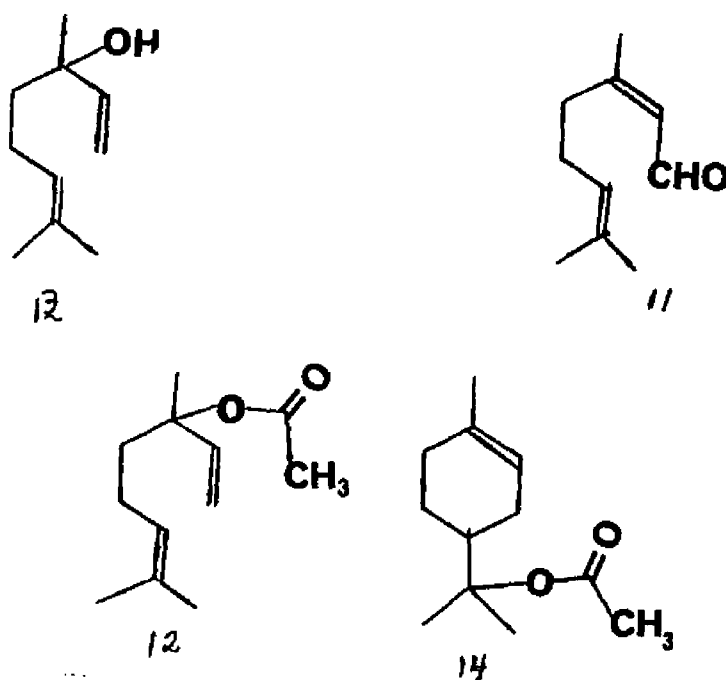
The methanol biting factor was shown to be isoquercitrin<sup>104</sup> (Figure 23).

Figure 23



It is interesting to note that isoquercitrin induces biting to a lesser degree than  $\beta$ -sitosterol. Citral (11), linalyl acetate (12), linalool (13), and terpinyl acetate (14) were isolated from the ether soluble material<sup>100</sup> (Figure 24). Subsequently, six small circles were inscribed on the circumference of filter paper at equal intervals. Each of these chemicals was put on cellulose powder and placed in the circles. Thirty newly hatched larvae were positioned in the center of the filter paper, and were attracted to all of these chemicals.

Figure 24

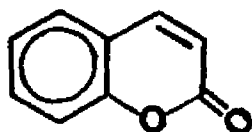


The swallowing factor consisted of more than one component;<sup>104</sup> in fact, maximum swallowing was attained when sucrose, cellulose powder, inositol, silica gel, and potassium phosphate were present in the diet. All of these components had varying potency effects as swallowing factors. Hamamura et al. suggested that inositol, sucrose, inorganic phosphate, and silica are co-factors of the swallowing factor which is cellulose powder. It is interesting to note that Hayashiya<sup>105</sup> in 1966 argued that flavones must have a hydroxyl group in the 2' or 3' position in order to evoke a biting response. His observations were reinforced when he showed that morin elicited a biting response. This flavone is not found in mulberry leaves, but rather it exists in the stem of Morus tinctoria.

Hamamura's bioassays were conducted on agar threads in order to test for the biting factor; the swallowing factor was quantitatively corroborated by counting the fecal pellets.<sup>100</sup>

In 1961, it was shown<sup>106</sup> that coumarin (Figure 25) was a food attractant of sweetclover weevils.

Figure 25

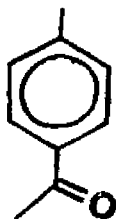


Traps baited with coumarin terminated the flight of the sweetclover weevil. This attractancy effect was subject to the time of the year in which the coumarin was tested with maximum attractancy arising over a four day period and then subsiding.

Saito and Munakata<sup>107</sup> in 1952 began their work on the isolation and structure elucidation of the food attractant of the rice stem borer larvae. On the assumption that newly hatched larvae could be used for biological assaying, they set about to extract air dried immature rice plants with ethanol, and subsequently, re-extracted the ethanol extract with diethyl ether. Biological assays indicated that the ether extract contained the active component. This extract was divided into three fractions: one,

acidic; the second, basic; the third, neutral. The neutral fraction possessed biological activity. By means of chromatographic techniques, they isolated 45 milligrams of a pure compound from 200 kilograms of dried rice plant. Using classical structuring methods, they concluded that the food attractant of the larvae was p-methylacetophenone (Figure 26).

Figure 26

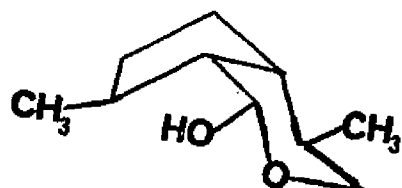


This compound was biologically active at  $10^{-6}$  micrograms. It was subsequently demonstrated, however, that whereas p-methylacetophenone was the food attractant for the larva, it could not be correlated with the food attractant of the moth.

Noting the observation made by Yano<sup>108</sup> that the fruit and leaves of Actinidia polygama were highly attractive to lacewings, Sakan isolated the active

components that evoked this attraction.<sup>109,110,111</sup> The neutral volatile fractions resulting from the methanolic extraction of leaves were thin layer chromatographed, and the chromatogram was subsequently exposed to adult male lacewings overnight. By noting bite marks on the chromatogram, an active band was established.<sup>111</sup> The steam distillate of the methanolic extract was vacuum distilled and shown to contain an extremely active hemi-acetal which was named neomatatabiol. The presence of the hemi-acetal with an R<sub>f</sub> value corresponding to that of the active compound was in very low concentration. Consequently, the isolation techniques were reset for large scale isolation of the active compound. After steam distillation of 1000 kilograms of leaves, several grams of crude attractant were obtained. Repetitive column chromatography of this substance resulted in the isolation of a small quantity of neomatatabiol (15) and isoneomatatabiol (16). By means of standard structural elucidation techniques, the structure of neomatatabiol was shown to be:

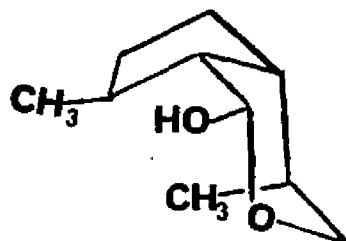
Figure 27



15

The second hemi-acetal isoneomatatabiol, was shown to have the following structure:

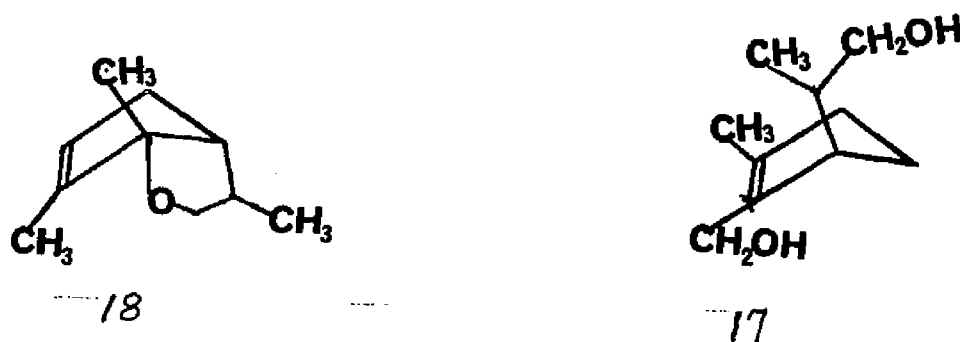
Figure 28



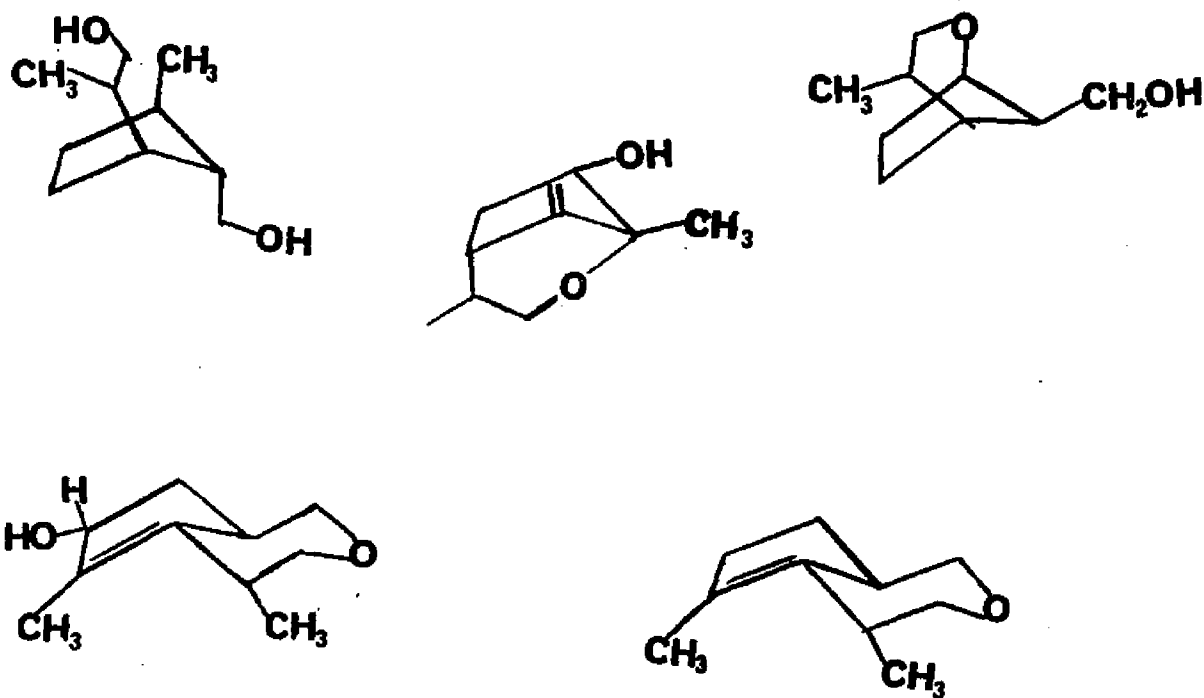
16

Positive biological response resulted when as little as  $10^{-6}$  micrograms were spotted on filter paper. As Butenandt noted in his studies of pheromones in Bombyx mori,<sup>24</sup> there was a gradient level of attractancy (vide supra); Sakan et al. demonstrated that this same gradient level exists in food attractants. For example, one microgram of iridodiol (17), 5-hydroxymatatabiether (18), 7-hydroxydihydromatatabiether (19), allomatatabiol (20) elicited biological activity. Only  $10^{-3}$  micrograms of metatabiol (21) and dehydroiridodiol (22) were needed to evoke a biological response.

Figure 29

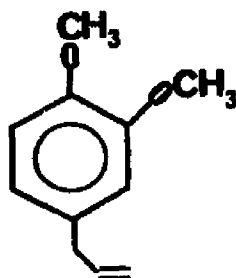






Kawano et al.<sup>112</sup> in 1968 identified the food attractant of the male oriental fruit fly from golden shower blossoms. Steam distillation of the petals, followed by chloroform extraction of the distillate, resulted in a crude material attractive to the fly. Gas chromatography of this active fraction, coupled with human nasal perception, tentatively identified the active compound as methyl eugenol (Figure 30).

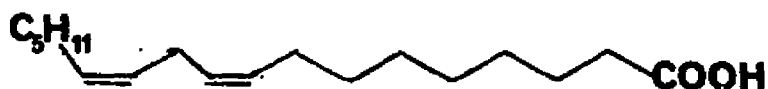
Figure 30



A comparison of the infrared spectrum of methyl eugenol with that of the chromatographically purified material confirmed their assumption.

In 1969, Hopkins et al.<sup>113</sup> reported the isolation and structural elucidation of what is believed to be a component of an attractant system for the honey bee. They extracted pollen with hexane and subsequently isolated the lipid fraction. This fraction was further separated by chromatographic methods. The structure of the bio-labile compound was proved by spectroscopic means to be octadeca-trans-2,cis-9,cis-12-trienoic acid (Figure 31).

Figure 31

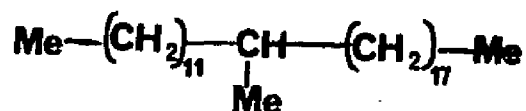


The bioassays were performed on 10 milligrams of pure acid applied to five grams of cellulose powder and placed in a room containing foraging bees. The number of visits were counted and compared to three controls containing only cellulose powder. The resulting 102 visits to the bait, as compared with 6, 10, 1 visits to the control, strongly suggested that the acid was responsible for attractancy.

In 1971, Jones et al.<sup>114</sup> published their results on the host seeking stimulant for the parasite of the corn earworm larvae. They isolated from 100 grams of earworm dry fecal matter the host attractant. Standard chromatographic and spectroscopic techniques enabled them to deduce the structure to be either 15-, 13-, 11-, 9-, or 7-methylhentriacontane. It was only through synthesis that the exact structure of the

host attractant was ascertained as 13-methyl hentriacontane (Figure 32).

Figure 32

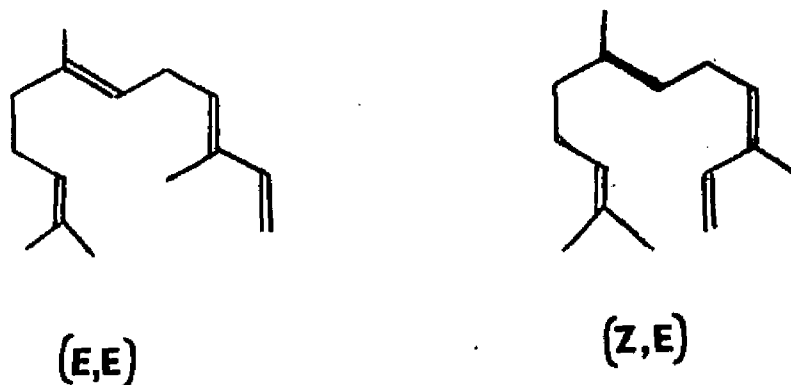


Biological assays showed that 13-methyl hentriacontane elicited responses at the 50 nanogram level. A gradient level existed for the methyl substituted  $\text{C}_{31}$  compounds. Activity diminished as proximity to the  $\text{C}_{13}$  position became remote. Jones suggests that retention of parasites in the field offers a means by which insects can be controlled.

The codling moth larva is a serious pest of temperature zone fruits. Sutherland and Hutchins<sup>115</sup> in 1973 published their findings of the food attractant for the newly hatched larvae. By standard isolation

methods of apple peels, they found that the active biological compound was  $\alpha$ -farnesene. (E,E) and (Z,E)  $\alpha$ -farnesene (Figure 33), two of the six possible stereoisomers, were shown to be biologically active at 100 nanogram quantities.

Figure 33



The remaining stereoisomers showed no bio-activity. Sutherland noted that the other four stereoisomers, which differ by virtue of spatial distribution and have no biological activity, lend credence to Amoore's theory of olfaction; i.e., the shape of the molecule is intimately associated with odor.<sup>16</sup> Additionally, it was observed that none of the stereoisomers exhibited any inhibitory activity, a phenomenon germane when dealing with sex attractants.

A history of the work which has been accomplished on sex and food attractants reflects the progress that has been achieved by devoted research in one of the most complex and tedious divisions of science; but it also suggests the plethora of work that remains to be done. Even after such work is accomplished, there is still no assurance that the insect problems which beset man will suddenly disappear. When Dr. Paul Muller received the Nobel prize for his work with DDT, it was recognized by many that his discovery was a great forward thrust in an effective control of pests through insecticidals. Since those early days, there is the strong suggestion that DDT has created a greater problem than the one it supposedly solved.<sup>141</sup>

Conceivably, the isolation and practical use of sex and food attractants may conjure up as many problems as those that have arisen with the discovery and use of DDT. Nevertheless, this work must continue if man intends eventually to assuage a critical ecology. The following experimentation is a move in this direction.

## CHAPTER II

### EXPERIMENTAL

The chemicals in this study were either reagent or spectroscopic grade. Unless otherwise specified, bioassays were performed in a 2'X2'X2' feeding bin at temperatures of  $26^{\circ}\text{C} \pm 3$ . The insects were maintained by Dr. A. Hammond, Department of Entomology, Louisiana State University, Baton Rouge. Because of seasonal changes, insect population varied from 1000-3000. The source of fresh sweet potatoes was the Department of Entomology of Louisiana State University; that of steam treated and alkali treated peels, the King Phaar Company of Oak Grove, Louisiana, and the Bruce Food Company of New Iberia, Louisiana, respectively. A strain of sweet potatoes resistant to weevil infestation was supplied by Dr. T. Hernandez of the Horticulture Department of Louisiana State University.

Nuclear magnetic resonance (NMR) spectra were obtained with a Varian A60A spectrophotometer. The Perkin-Elmer Model IR-137 Double Beam Spectrophotometer provided infrared spectra, while uncorrected melting

points were acquired from Fisher-John apparatus. Infrared data are reported in microns ( $\mu$ ); NMR, in parts per million (ppm) with TMS as 0 ppm. Some of the spectrograms, obtained from a Varian M-66 Double Focusing Mass Spectrometer, were provided by Ms. Cheryl White, Scientific Research Specialist, Department of Chemistry, Louisiana State University, Baton Rouge; others were acquired from Dr. D. Durst, Department of Chemistry, University of New York at Buffalo. Ms. Paula Watts, Scientific Research Specialist, Department of Chemistry, Louisiana State University, Baton Rouge, and Mr. Paul Moses, graduate student, Louisiana State University, Baton Rouge, performed the gas chromatography-mass spectrometry on a Perkin-Elmer 990 Gas Chromatograph with a Flame Ionization Detector, containing a stream splitter and Belman-Watson Porous Pyrex Separator. A Hitachi Perkin-Elmer RMS-4 Mass Spectrometer was used.

All elemental analyses were performed by Mr. Ralph Seab, Scientific Research Specialist, Department of Chemistry, Louisiana State University, Baton Rouge. Germane spectra of compounds previously synthesized by Dr. L. Green, Gulf South Research Laboratories, New Iberia, Louisiana, and Dr. C. Aaron, visiting scientist



in the Department of Chemistry, Louisiana State University, Baton Rouge, were used for comparison.

1. Concentration of fecal extract and bioassay

A hexane extract of fecal material from virgin female sweet potato weevils which had previously been shown to possess sex attractancy activity was decanted and the residual fecal pellets were re-extracted with hexane. The hexane solutions were combined and evaporated by a gentle nitrogen stream until a light orange yellow material resulted. This residue was shown to have a high level of sexual attractancy, which was noted by hand holding the open bottle containing the residue in a feeding bin. Within seconds, the normally phlegmatic weevils began to move agitatedly. Subsequently, male insects were observed flying in the direction of the bottle.

2. Bioassay of hexane extract of frass

A drop of the hexane extract from the fecal pellets of the virgin sweet potato weevils was spotted on a piece of Whatman #1 filter paper. The hexane was allowed to evaporate and the filter paper was then placed into the insect feeding bin. Within 20-30 seconds after the filter paper had been inserted into the bin,

males were observed in flight. Almost immediately, a number of the flying insects lighted upon the paper itself. This means of observing the sexual evoking power of samples to be tested comprised the bioassay for sexual attractancy. Bioassays were best carried out in the latter part of the afternoon.

3. Silicar thin layer chromatography of hexane extract

After having tried various elution systems, fifty milligrams of the residue resulting from a hexane extract of the fecal pellets were placed onto a Malinkroft 7G thin layer silicar plate and chromatographed with benzene-ethyl acetate (98:2). After developing the plate and evaporating the solvent from it, the entire chromatogram was positioned in the insect feeding bin. Observations were made to ascertain if any biological activity was elicited. None was discerned. The silicar was then scraped from the plate and extracted with 200 milliliters of hexane. The hexane extract was concentrated and tested for biological activity. There was none.

4. Alumina thin layer chromatography of hexane extract

Fifty milligrams of the residue resulting from the fecal extract of virgin females were placed

onto an alumina thin layer plate. The plate was developed by means of a benzene-ethyl acetate (98:2) solvent system. The solvent was evaporated and the entire plate was put into a feeding bin to check for biological activity. No biological response was observed while the material was on the thin layer plate. Subsequently, the plate was thoroughly extracted and the solvent was reduced in volume by nitrogen gas stream. The remaining solvent was then assayed for biological response. No activity was noted. Later it was demonstrated that an active hexane extract would lose all activity immediately if a few drops were placed onto an adsorbent surface such as sand, alumina, or silicic acid.

##### 5. Base and acid treatment of fecal extract

One milliliter of the fecal extract was added to a 50 milliliter Erlenmeyer flask equipped with a magnetic stirring bar. Twenty-five milliliters of a 6N ethanolic sodium hydroxide solution was poured into the stirring solution. The solution was stirred for three hours, whereupon the stirring was terminated and an aliquot of 4 milliliters was removed. This aliquot was tested for biological activity. When the insects were exposed to a sample of this material,

they commenced flying. At the same time, one milliliter of the hexane extract from fecal pellets was placed into a 50 milliliter flask equipped with a stirring bar. To this was added 25 milliliters of a 6N solution of hydrochloric acid. The solution was allowed to stir for 3 hours, after which an aliquot was withdrawn and bioassayed. Again, upon exposing the insects to a sample of this material, they began to fly.

#### 6. Sublimation of fecal extract

Approximately 200 milligrams of the residue in hexane from the hexane extract of the sweet potato weevil frass was put into the bottom of a subliming apparatus. The hexane was evaporated by a gentle stream of nitrogen gas. Then the cold finger containing dry ice and acetone was affixed. A vacuum, 2 Torr, was pulled for thirty minutes before heating commenced. The temperature of the oil bath was raised slowly until a maximum of 140°C was attained. At this temperature the residue was sublimed for three hours. Subsequently, the heating bath was removed and the dry ice acetone cold finger was allowed to warm to room temperature. The vacuum was released and the cold finger was removed and tested for biological activity. This bioassay proved

very active. Additionally the sublimation residue was checked for biological response. It did show some activity, but to a lesser extent than that of the sublimate. The residue was shown to be thermally stable at a temperature of 170°C.

7. Mass spectroscopy of sublimed fecal material

After three sublimations, each using two hundred milligrams of fecal extract, the cold finger was washed with 10 milliliters of hexane. The hexane was then evaporated by a stream of nitrogen gas. There remained a white waxy material eliciting a high level of biological activity from the sweet potato weevil when bioassayed. This material was placed in a capillary tube and subjected to mass spectral analysis. The resulting spectrum revealed nothing more than the faint peaks of a sample that had previously been run on acetylene compounds.

8. Extraction of whole weevils and bioassay of the extract

One hundred and twenty-seven thousand frozen sweet potato weevils of mixed sex were placed in a 2000 milliliter, three necked, round bottom flask equipped with a stirring bar, a nitrogen inlet valve, and a

West condenser fitted with a drying tube containing calcium chloride. Into this flask were added 600 milliliters of methylene chloride. The insects were macerated for 48 hours under nitrogen. The solution was then vacuum filtered, and the filtrate was flash evaporated at less than 35°C. A bioassay of the resulting concentrate proved biologically inactive. The concentrate was stored in a freezer for further use. Subsequently, the remaining weevils were continuously extracted in a Soxhlet apparatus with diethyl ether for 24 hours. The extract was collected and flash evaporated at less than 30°C. There resulted a yellowish oil which was tested for biological activity. No activity was noted.

9. Attempted milking of virgin females by a  
modified Yamamoto technique<sup>86</sup>

Approximately 500 virgin female sweet potato weevils were placed into a 1000 milliliter Buchner flask containing cubes of sweet potato. An inlet Tygon tube was affixed through which air, filtered by a potassium hydroxide trap, was streamed over the insects. An outlet Tygon tube, passing into a 3A molecular

sieve bed, was attached to the Buchner flask. From the 3A molecular sieve bed, a Tygon hose was directed into a dry ice acetone trap. The insects were milked in this fashion for 24 hours. A large amount of water collected in the dry ice acetone trap, whereupon the milking process was terminated and the cold trap, removed. Immediately, the ice was extracted three times with 100 milliliters of methylene chloride. The methylene chloride was concentrated by flask evaporation and tested for biological activity. None was detected. The molecular sieve was then extracted with methylene chloride. The extract was concentrated and tested for biological response. It too elicited no activity.

10. Chemicals tested for biological activity

geraniol	phenol
citral	p-cresol
linalool	pentanoic acid
geranoic acid	benzoquinone
Chloroseptic Mouth Wash	resorcinol
Gleem Tooth Paste	octanol
Ole Spice After Shave	valeric acid
Lifebouy Soap	acetaldehyde

dodecanoic acid	3-heptanone
tetradecanoic acid	myrcene
hexadecanoic acid	methyl tetradecanoate
octadecanoic acid	methyl pentadecanoate
heptanal	methyl hexadecanoate
octanal	methyl heptadecanoate
n-hexadecanol	methyl octadecanoate
2-heptanone	tricosane

11. Weevil preference for pith or skin surface

Two sweet potatoes were halved and placed into the feeding bin to ascertain if the insects showed a preference for either the pith or the skin. After 20 hours in the bin, the potato halves were removed and checked for puncture marks, indices of feeding response. No evidence of biting was observed on the exposed pith surface; however, there was evidence that the weevils had been eating on the skin surface. It was noted that the weevils congregated on the tenebrous side of the potato.

12. Weevil exposition to the pith alone

The pith of a sweet potato was excised by a paring knife. After being sectioned, this pith was placed in a feeding bin for 24 hours. Subsequently, the sections were removed and checked visually for



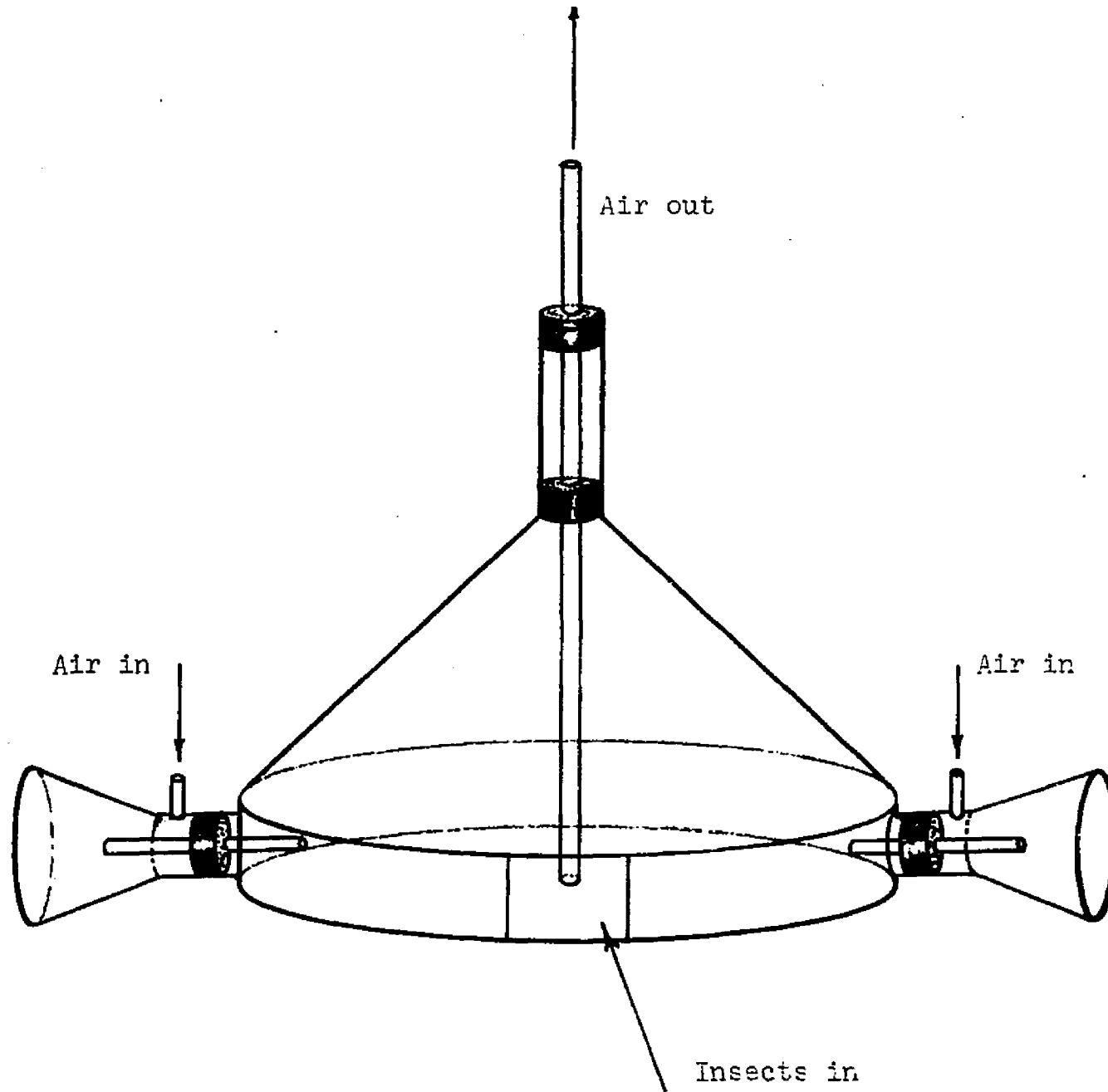
evidence of feeding. No feeding response was noted. Two cubes of pith were then placed, respectively, into three cylindrical cardboard pint containers. Fifty sweet potato weevils of mixed sex were inserted into each container for 24 hours, whereupon the cubes were withdrawn and assayed for biological response. No feeding punctures were visually observable.

13. Weevil exposition to the skin alone

The skin of five sweet potatoes weighing approximately 1.5 grams was placed on a towel and inserted into the feeding bin containing approximately 1500 weevils. After 24 hours, the skin was removed and checked for insect infestation. There were 10-15 insects embedded in the skin.

14. Olfactometry tests of skin versus potato cube

Into an olfactometry vial were placed one-half grams of sweet potato skin. The vial was subsequently affixed to an olfactometer (See Illustration). At the same time, a sectioned sweet potato was inserted into a control vial. An olfactometry test was conducted with fifty insects of mixed gender. After 24 hours, the test was terminated and the insects counted. The vial with the potato skin had nine insects; the one with the cube, only eight.



OLFACTOMETER

This experiment was repeated five times; each time the results were similar.

15. Olfactometry tests of pith blends

Nine hundred grams of sweet potato piths from three sweet potatoes together with water and ice were blended. The blend was poured into a Buchner funnel and vacuum filtered. The filtrate was collected and poured into a bottle; the residue was also bottled, and both containers were biologically assayed. Biological tests were conducted by means of olfactometry. Of fifty weevils involved in this test, five were drawn to the filtrate; eight, to the residue.

16. Olfactometry tests of skin blends

Six grams of skin from 20 potatoes were blended with water and ice. The resulting blend was poured into a Buchner funnel and vacuum filtered. Both the filtrate and the residue were bottled separately and biologically assayed by means of olfactometry. Fourteen of the 50 weevils were drawn to the filtrate; nine, to the residue.

17. Olfactometry tests of peel blends

Four hundred and eighty grams of peels from 10 potatoes, i.e., skins plus a small amount of pith were blended with ice and water. Blending was continued

until the mixture was somewhat uniformly meshed. At this time, the mixture was vacuum filtered. The filtrate and the residue were bottled separately and biologically assayed by means of olfactometry. Out of 50 weevils, 11 went to the filtrate; 8, to the residue.

18. Olfactometry tests of piths blended with chloroform

One and a half kilograms of sweet potato pith were placed into a Waring blender with chloroform. After it was blended, the mixture was poured into a Buchner funnel and vacuum filtered. The chloroform filtrate, an orange yellow color, was flash evaporated at 30°C. The resulting brownish red residue was tested in an olfactometer to determine if any attractancy existed. Of the 50 insects tested, 4 were drawn to the test material; 9, to the control vial; 37 were unresponsive. The test was repeated a number of times with similar results.

19. Olfactometry of steam distilled peels

Into a 2 liter round bottom flask were placed approximately 500 grams of peels. The peels were steam distilled for 5½ hours. The resulting distillate was

collected in a series of ice cooled Erlenmeyer flasks. The contents of the flasks, possessing a pungent vegetable odor, were decanted into a bottle and biologically assayed against a potato cube by means of olfactometry. Of 50 weevils, 16 preferred the distillate; 8, the cube. The pulp remaining after steam distillation was collected. After partial drying by vacuum filtration, it was biologically assayed and found inactive.

20. Olfactometry of lypholyzed methylene chloride-methanol extracted skin

Approximately 30 grams of lypholyzed skins of the sweet potato were placed into an extraction thimble which was subsequently put into a Soxhlet extraction apparatus. The skins were continuously extracted with a mixed solvent of methylene chloride and methanol (50:50) for 20 hours. The resulting reddish extract was inserted in a freezer for 12 hours, whereupon a white material precipitated. The cold solution was then removed and vacuum filtered. The residue was washed with two 50 milliliter portions of cold solvent. The residue was a white amorphous material, having a melting point of 98-103°C. Upon melting, it acquired a reddish hue.

The filtrate was then flash evaporated.

There remained a reddish brown gum which was dissolved in 25 milliliters of methylene chloride. Both the reddish brown and white residue were bioassayed. Of 50 weevils, 8 preferred the brown; 6, the white.

21. Olfactometry of acetylated white precipitate  
from extraction of lypholyzed skin

One gram of the white material that had precipitated after cooling of the methylene chloride methanol extract of the lypholyzed skin was placed into a 100 milliliter round bottom flask containing a magnetic stirring bar. To this white material was added 20.4 grams (0.2M) of acetic anhydride and 2 milliliters of pyridine. The solution was allowed to stir at room temperature for 27 hours; then 30 milliliters of ethanol were dripped into the flask. The reaction was allowed to stir for an additional 40 minutes with cooling. The contents of the reaction flask were subsequently poured into a beaker which was put into a freezer. After four hours, the beaker was removed and the precipate was vacuum filtered. The resulting residue was waxy, amorphous, and pale yellow with a melting point of 72-75°C. An olfactometric bioassay proved negative.

22. Insect response to piths treated with residue of extract from lypholyzed skin

The piths of two halves of sweet potatoes were painted with a small portion of the residue resulting from evaporation of the methylene chloride-methanol extract of the lypholyzed skin. The halved potatoes were placed into a feeding bin and allowed to remain there for 24 hours; whereupon they were removed from the bin and visually checked for feeding response. There were multiple bites on the potato halves. This experiment was repeated three times; each time results were positive.

23. Boxed-insect response to corks painted with residue of extract from lypholyzed skin

Approximately one milligram of the residue from the concentrate of the methylene chloride-methanol extract of lypholyzed skin was painted on two number 10 corks. These corks were positioned in a pint size cylindrical cardboard container. Two additional corks treated with methylene chloride-methanol were placed in the same container. Finally, 15 weevils of mixed sex were added to the container which was sealed and situated in a quiet place. After 24 hours, both the test and control corks were removed from the

container and checked visually, then, with the aid of an adjustable 3X dissecting microscope. No evidence of biting was observed on any of the corks.

24. Binned-insect response to corks painted with residue of extract from lypholyzed skins

Two number ten corks were painted with 2.4 milligrams of the concentrated filtrate from the methylene chloride-methanol extract of the lypholyzed skin. The corks were placed into a feeding bin. After 21 hours, the corks were removed and checked visually. Observation of the corks under a 3X dissecting microscope indicated that one cork had more than 380 bites; the other, more than 410 bites.

25. Preparation of a stock solution of the extract of lypholyzed skin

Twenty-eight grams of lypholyzed skin were continuously extracted in a Soxhlet extractor with methylene chloride-methanol for 24 hours. The solution was then placed into a freezer for an additional 24 hours. At the end of this time, a white material had precipitated. The solution was vacuum filtered; the precipitate was collected and stored. The filtrate was flash evaporated at 34°C. The resulting brownish



red residue weighing 0.51 grams was diluted with 110 milliliters of methylene chloride. This constituted the stock solution for further experiments.

26. Test of stock solution on binned corks

0.5 milliliters from the stock solution of experiment 25--the equivalent of 2.4 milligrams of residue--was applied to 5 number 10 corks. The test corks plus 5 number 10 corks treated with one-half milliliter of pure methylene chloride were all labelled and randomly positioned on a fiber glass testing tray. This tray was placed into a feeding bin at 11 A. M. and remained there for 21 hours. At 8 A. M. the following morning, the corks were removed and inspected for puncture marks under a 3X dissecting microscope. Only the test corks evinced a feeding response. The feeding level was approximately 400 bites per cork. This experiment was repeated more than 50 times, always with the same results.

27. Biting frequency as a function of dilution

Two 1 milliliter aliquots of the stock solution from experiment number 25 were diluted tenfold and a hundredfold, respectively. To each of five number 10 corks was applied one-half milliliter of the tenfold

dilution. These five corks plus five control corks treated with one-half milliliter of pure methylene chloride were randomly placed on a testing tray which was positioned into a feeding bin. After 21 hours, the tray was removed and checked under a dissecting microscope for feeding responses. The bites ranged from 50-100 on the tests corks; the control corks showed no evidence of biting. Next, five number 10 corks were painted with one-half milliliter of the hundredfold dilution. Again, these five corks in addition to five control corks treated with one-half milliliter of methylene chloride were put in the tray at random, and inserted into the one-half feeding bin. After 21 hours, the corks were removed and inspected for biological activity under a dissecting microscope. The feeding response on the test corks ranged from 1-5 bites; the control corks evinced no biting.

28. Acid treatment of the stock solution and bioassay

Four milliliters of the stock solution from experiment 25 were added in a 50 milliliter Erlenmeyer flask containing a magnetic stirring bar. Subsequently, 25 milliliters of 6N hydrochloric acid were inserted; the flask was capped and allowed to stir for three hours. The content was then poured into a separatory

flask and the organic layer separated. The water layer was washed with two 25 milliliter portions of methylene chloride. The organic layers were combined and flash evaporated. To the resulting residue were added four milliliters of methylene chloride. A half milliliter of this methylene chloride was painted on eight corks which were placed into a feeding bin for 21 hours. At the end of this time, the corks were removed and examined for evidence of feeding response. None was noted. Two milliliters of the water layer were tested for biological activity. They also proved negative. Additionally, a two milliliter aliquot of the water was brought to pH 7 and also bioassayed. The response was negative. The tests were repeated with 3N and 1N hydrochloric acid, and again the bioassays were negative.

29. Alkali treatment of the stock solution and bioassay

Four milliliters of the stock solution from experiment 25 were added to a 50 milliliter Erlenmeyer flask containing a magnetic stirring bar. Subsequently, 25 milliliters of 6N sodium hydroxide were added, and the mixture was allowed to stir for three hours. The content was then poured into a separatory flask and

the organic layer separated. The water layer was washed with two 25 milliliter portions of methylene chloride. The organic layers were combined and flash evaporated. To the resulting residue were added four milliliters of methylene chloride. One-half milliliter of this methylene chloride was painted on eight corks which were placed into a feeding bin for 21 hours. At this time, the corks were removed and inspected for evidence of feeding response. None was noted. Two milliliters of the water layer were tested for biological activity. They also proved negative. Additionally, two milliliters of the water was brought to pH 7 and bioassayed. Response was negative. The tests were repeated with 2N and 1N sodium hydroxide; again the bioassays were negative. The basic test was also conducted in a round bottom flask under a nitrogen gas environment. The bioassays of the resulting organic layers proved negative. An aliquot of the basic water was then bioassayed. The result was negative. An aliquot of the neutralized water layer was bioassayed. It too was negative.

30. Bioassay of alkali treated potato peels  
adjusted to pH 6

A liter of five gallons of alkali treated potato peels, pH 12, obtained from Bruce Foods Corporation of New Iberia, Louisiana, was titrated with 6N hydrochloric acid until the pH was adjusted to approximately 6. A methylene chloride extraction was attempted; whereupon an emulsion resulted requiring centrifugation at 7000 RPM's for one hour. At this time, the emulsion was dissipated and a disposable pipette was used to withdraw the top aqueous layer from the centrifuge tubes. A portion of this aqueous layer was tested for biological activity. The bioassay consisted of treating each of five number 10 corks with a milliliter of the aqueous layer. These corks were placed in a feeding bin for 21 hours. At the end of this time, one of the corks exhibited a few bites when inspected under a dissecting microscope. The test was repeated with 10 corks treated with one milliliter of the aqueous layer. This time, however, there were no discernible bites. The organic layer that remained in the centrifuge tubes was decanted into a round bottom flask and flash evaporated at less than 34°C. The resulting residue was dissolved in

two milliliters of methylene chloride and tested for biological activity on four number 10 corks. No response was observed.

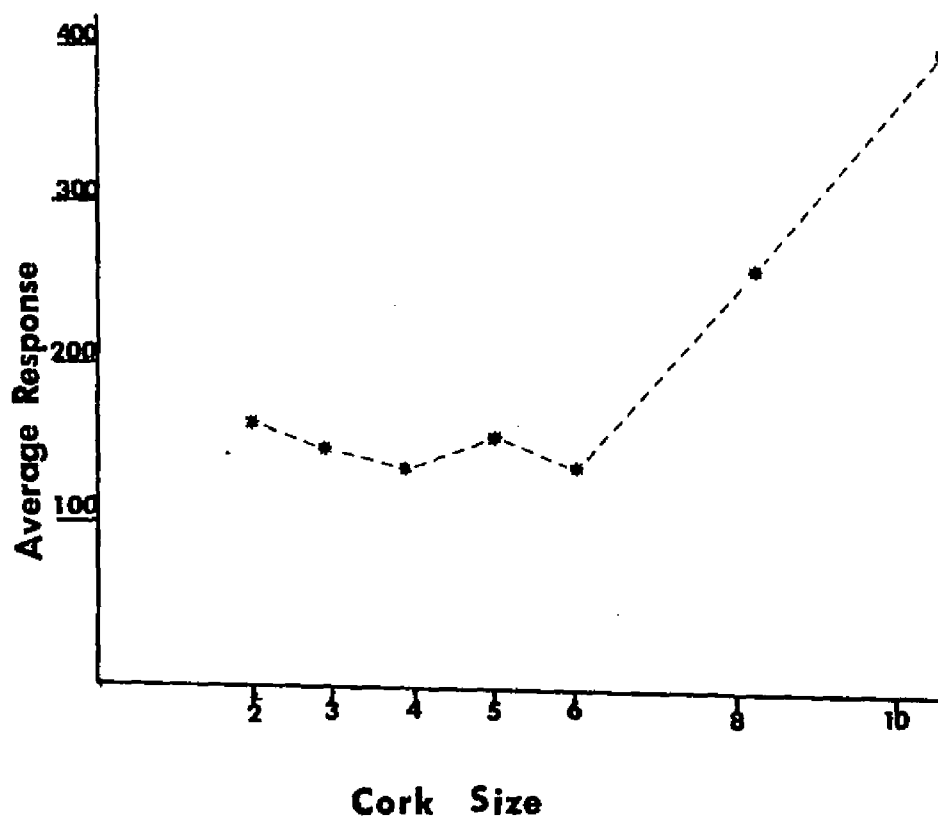
31. Bioassay of insect feeding response in varying spatial environments

Eight number 10 corks were treated with one-half milliliter of the stock solution from experiment 25. Two of these corks were placed into a pint, quart, half gallon, and gallon cardboard container, respectively. To each container was added 25 weevils and two cubes of potato. These cartons were capped for 24 hours; whereupon the corks were removed and checked for feeding response. No punctures were observed. The test was repeated with the same results.

32. Bioassay of insect feeding response to varying surface sizes

Five corks of varying sizes, i.e., numbers 2, 3, 4, 5, 6, 8, and 10, were each painted with one-half milliliter of the stock solution from experiment 25. Each category of corks plus five control corks of the same size were placed into a feeding bin and left there for 24 hours. At the end of this time, the corks were checked for biological response. The control corks

exhibited no puncture marks; however, Graph 1 illustrates the feeding response on the treated corks.



Graph 1

33. Bioassay of stock solution applied to various vegetables

One milligram of the residue obtained from the stock solution of experiment 25 was respectively applied

to green beans, cucumbers, kidney beans, pinto bean diet, eggplants, Whatman number one filter paper, Whatman number one filter paper wrapped around 2% agar, and the surface of 2% agar within a Petri dish. All of the test materials were placed on paper towels and positioned into the feeding bin for 21 hours. Subsequently, these materials were removed and checked for feeding response under a 3X dissecting microscope. No activity was observed. This experiment was repeated with 2.3 milligrams and 4.6 milligrams of the residue. Again, there was no positive response in either case.

34. Bioassay of stock solution on paraffin surface

Ten number 10 corks were immersed in melted paraffin. The corks were removed and the wax was allowed to congeal. Five of the corks were treated with one-half milliliter of the stock solution from experiment 25; the other five were treated with one-half milliliter of pure methylene chloride. All 10 corks were randomly placed on a testing tray and inserted into a feeding bin for 21 hours. At the end of this time, the corks were removed and microscopically checked for feeding response. The five test corks showed an average biting response of 50, whereas the control corks showed no biting response.



35. Bioassay of stock solution on resistant strain  
sweet potato

Two W-15-2 variety sweet potatoes resistant to weevil infestation were demarcated into halves. One half of each potato was painted with one milliliter of the stock solution from experiment 25; the other half was treated with one milliliter of pure methylene chloride. These potatoes were placed into a feeding bin for 21 hours. After this time it was noted that the potatoes were infested with weevils on the test sides; no weevils were observed on the control side. When the potatoes were removed and inspected under a microscope, it was observed that there were multiple feeding responses on the test halves; the control side did not appear to have been eaten. However, the feeding response, unless manifold, was difficult to discern on such a rough surface. Additionally a cruciform was painted on one of the resistant strain potatoes with one milliliter of the stock solution from experiment 25. This potato was positioned in a feeding bin for 21 hours. At the end of this time, the potato was observed while still in the feeding bin. It was noted that some of the insects had amassed themselves on the potato in the shape of a cross at the painted site.

The potato was subsequently removed from the bin and checked microscopically for feeding response. The feeding was heavy along the lines of the cross.

36. Chromatography of the concentrated filtrate from the extracted lypholyzed skin and bioassay

Approximately five hundred milligrams of the residue obtained from continuous extraction of the lypholyzed skin were dissolved in methylene chloride and applied to a two millimeter plate of silica 7GF. The plate was eluated by a solvent system consisting of petroleum ether, benzene, ethyl acetate (90:5:5). Five bands were shown to be present by means of both long and short ultraviolet light, and also by means of positioning the plate into an iodine chamber. The chromatography run was then repeated with an additional quantity of residue. This time the plate was not positioned into an iodine chamber. Both plates were put into the feeding bin for 21 hours. At the end of this time, the plates were removed and inspected with a dissecting microscope for feeding punctures. No response was observed. Subsequently, an additional plate was spotted with residue and developed. Five bands were scraped from the plate and each was extracted with methylene chloride-methanol (50:50). The organic

solution was concentrated. Five number 10 corks were labelled 1-5 and the corresponding extracts from the chromatographic bands were applied. The corks were then positioned into the feeding bin and remained there for 21 hours. At the end of this time, the corks were removed and inspected for biological response. Band number two showed approximately 180 bites; band number one showed one bite; the remaining bands were negative. The chromatography procedure and bioassay were repeated with similar results; however, band number one had no bites.

### 37. Bioassay in transparent plastic cages

Each of three corks was painted with one-half milliliter stock solution from experiment 25. These, together with three control corks treated with methylene chloride only, were placed equidistant from two water sponges in a transparent plastic cage containing 300-400 weevils. The cage was then sealed with tape and positioned into a controlled environment. It remained there for 24 hours and was subsequently removed. The corks were taken out and checked for feeding response by means of a dissecting microscope. The test corks demonstrated an average of 280 bites; the control corks showed no feeding response. This experiment was

repeated four times with similar results. The average feeding response ranged from 200-300 bites.

38. Time study of feeding response

At 12 noon, three corks painted with one-half milliliter of stock solution from experiment 25 and three control corks treated with pure methylene chloride were positioned equidistant from two water sponges in a transparent plastic cage containing 300-400 weevils. The plastic containers were sealed and inserted into a controlled environment for 12 hours; whereupon the cages were removed and the corks withdrawn to be checked for feeding response. Observations revealed that the test corks had an average response of 270 bites; the control corks had none.

At 12 midnight, three corks painted with one-half milliliter of stock solution from experiment 25 and three control corks treated with pure methylene chloride were positioned equidistant from two water sponges in a transparent plastic cage containing 300-400 weevils. The plastic containers were sealed and inserted into a controlled environment for 12 hours; whereupon the cages were removed and the corks withdrawn to be checked for feeding response. Examination of the corks

revealed that the test corks had an average response of 26 bites; the control corks had none. Both experiments were repeated with similar results.

39. Bioassay of sublimate and sublimate residue

Five hundred milligrams of the residue of the concentrated filtrate from the extraction of lypholyzed skins were placed into the bottom of a sublimator flask. A finger was subsequently affixed and a vacuum, 2 Torr, was pulled for one hour, at which time the vacuum was broken. The finger was then removed and thoroughly cleaned. It was re-affixed, the vacuum was restored, and dry ice in acetone was added to the finger. The bath temperature was slowly incremented until a final temperature of  $141^{\circ}\text{C}$  was attained. At this temperature, sublimation was continued for eight hours; whereupon the oil bath was removed and the cold finger allowed to warm to room temperature. The finger was then removed and washed with five milliliters of methylene chloride and methanol (50:50). Next, the solvent was evaporated by a stream of nitrogen gas and the sublimate weighed. It weighed 1.3 milligrams. Subsequently, the sublimate was redissolved and painted onto a number 10 cork. This cork was placed into a feeding bin for 21 hours. At the end of this time, the cork was removed and inspected

under a microscope for feeding response. There were 258 bites. The residue from the bottom of the sublimator was dissolved in five milliliters of methylene chloride and methanol (50:50). Five number 10 corks were each painted with one-half milliliter of this dissolved residue. The corks were then placed into a feeding bin for 21 hours; whereupon they were removed and checked for feeding response. Four corks showed no bites; one cork showed four bites. Both tests were repeated with similar results; however, none of the corks painted with the sublimate residue showed a feeding response.

#### 40. Bioassay of steam treated peels

Thirty gallons of steam treated peels, obtained from King Phaar Corporation of Oak Grove, Louisiana, were frozen. Three liters of these frozen peels were lypholyzed. The pulp resulting from lypholyzation was placed in a large Soxhlet extractor and extracted with methylene chloride and methanol (50:50) for 24 hours. The resulting solution was inserted into a freezer for 24 hours. At the end of this time, the precipitate was filtered and the filtrate, flash evaporated at 34°C; a red oil remained. One hundred milligrams of this oil were dissolved in 25 milliliters of methylene

chloride. Five number 10 corks were each treated with one milliliter of this test solution and placed into the feeding bin for 21 hours; whereupon they were checked by microscope for feeding response. There was an average of six bites per cork. The bioassay was repeated, using five corks, respectively painted with 50 milligrams of the red oil dissolved in one milliliter of methylene chloride. The biting frequency averaged 85 per cork. The precipitate that had formed in the freezer was checked for biological activity. None was apparent.

41. Bioassay of sublimate and sublimate residue of King Phaar peels

One gram of the red oil resulting from the Soxhlet extraction of the King Phaar peels was put into a sublimator flask, a finger was affixed, and a vacuum, 2 Torr, was pulled for one hour. At this time the vacuum was broken. The finger was then removed and thoroughly cleaned. It was reaffixed, the vacuum was restored, and dry ice in acetone was added to the finger. The bath temperature was slowly incremented until a final temperature of  $141^{\circ}\text{C}$  was attained. At this temperature, sublimation was continued for eight hours; whereupon the oil bath was removed and the cold finger allowed

to warm to room temperature. The unctious material on the finger was then removed with five milliliters of methylene chloride and methanol (50:50) and placed into a 10 milliliter beaker. The solvent was evaporated by a stream of nitrogen gas and the contents of the beaker were weighed. The weight was 11.3 milligrams. These 11.3 milligrams were then redissolved in three milliliters of methylene chloride, one milliliter of which was painted on each of three corks. These three corks, plus three control corks treated with pure methylene chloride, were inserted into a feeding bin for 21 hours. At the end of this time, the corks were removed and checked for feeding response. The test corks averaged four bites; the control corks, none. The residue in the sublimator was dissolved in 25 milliliters of methylene chloride. Five corks were each painted with one milliliter of this solution. These five corks, in addition to five control corks treated with pure methylene chloride, were positioned in a feeding bin for 21 hours; whereupon they were removed and inspected for feeding response. The average feeding response on the test corks was four bites; the control corks showed none.



One gram of the red oil was again sublimed under the same conditions. Five milligrams of the sublimate in two milliliters of methylene chloride were mixed with five milligrams of the sublimator residue in four milliliters of methylene chloride. Each of five corks was painted with one milliliter of this solution. These five corks, together with five control corks, were placed in a feeding bin for 21 hours; whereupon they were removed and inspected microscopically for a feeding response. Each test cork contained approximately eight bites; the control corks evinced none.

42. Gas chromatography and mass spectra of  
sublimates of lypholyzed skin (polyethylene  
beaker)

Two sublimations were conducted on the concentrated filtrate from the extraction of lypholyzed skin of the potato. One of these was collected in a polyethylene disposable beaker and the volume of the solvent, reduced by a nitrogen gas stream. The other was used to optimize gas chromatographic separation. It was found that a 15% diethylene glycol succinate column, preconditioned for two days at 200°C, was most effective. The most felicitous gas chromatographic program consisted of an initial temperature of 80°C with

a six minute hold, followed by a  $2^{\circ}\text{C}$  per minute increment, and terminated with a  $190^{\circ}\text{C}$  temperature with an infinite hold. A carrier gas flow rate of 27 milliliter per minute produced optimum results. The first sublimate, which had been stored in a polyethylene disposable beaker, was subjected to gas chromatography coupled with mass spectroscopy. Spectra were obtained from two prominent peaks. The first of these two peaks was analyzed as 2,6-di-tert-butyl-4-methylphenol. An analysis of the second corresponded to phenol (vide end of this section for spectra). A bioassay on five milligrams of phenol proved positive; the biting frequency on five corks averaged 300 per cork; however, the puncture marks were larger than normal. The bioassay was repeated four times with similar results. Bioassays at the level of 0.5 milligrams showed an average biting frequency of 22; at the level of 0.05, an average biting frequency of one; at the level of 50 milligrams, an average biting frequency of three. When the phenol applicator was placed near the insects, they did not move; yet, their antennae appeared to be either stunned or exacerbated.

43. Gas chromatography and mass spectra of  
sublimates of lypholyzed skin

Five sublimates were conducted on five hundred milligrams of the concentrated filtrate from the extraction of lypholyzed potato skins. The sublimates were collected in a glass vial and the solvent was removed by a stream of nitrogen gas. Gas chromatography and mass spectra were performed (vide supra). Twelve compounds were tentatively structured. The spectra of the following compounds are found at the end of this section: methyl tetradecanoate, methyl pentadecanoate, methyl hexadecanoate, methyl heptadecanoate, methyl octadecanoate, methyl octadecdienoate, methyl octadectrienate, methyl docosanoate, methyl eicosanoate, n-heneicosane, n-tricosane, and squalene. The entire procedure was repeated with similar results. The experiment was again repeated starting with a concentrated filtrate resulting from methylene chloride-ethanol (50:50) of 30 grams of lypholyzed skin. The resulting mass spectra did not show the presence of methyl esters; no parent masses could be discerned because of the aliphatic nature of the compounds.

44. Bioassay of compounds identified through mass spectroscopy

All 12 tentatively identified compounds were bioassayed to ascertain if any elicited a feeding response. The first of these, methyl tetradecanoate, was biologically tested in the following manner: each of three corks was painted with four milligrams of the sample dissolved in one-half milliliter of methylene chloride. The test corks, plus three control corks, were placed equidistant from two water sponges in a plastic cage containing 300-400 weevils. The cage was sealed and put into a controlled environment for 21 hours. At the end of this time, the corks were removed from the plastic container and inspected microscopically for feeding response. Neither the test nor control corks evoked a feeding response. This same procedure was repeated with the remaining 11 compounds. The results were always identical. A mixture consisting of one milligram of each of the 12 compounds was bioassayed. It showed no biological activity. The esters were then hydrolyzed and bioassayed. Again, there was no feeding response.

45. Extraction of the lypholyzed skin with  
methylene chloride

Thirty grams of lypholyzed potato skins were placed into a Soxhlet thimble which was then inserted into a Soxhlet extractor. The skins were extracted by methylene chloride alone for a period of 21 hours. Subsequently, the solution was collected and placed in a freezer for 21 hours; whereupon it was removed, vacuum filtered, and flash evaporated at 34°C. The residue was dissolved in a small quantity of methylene chloride and poured into a sublimator. The solvent was evaporated by means of a stream of nitrogen gas. A vacuum, 2 Torr, was then pulled for one hour, at which time the finger was removed and thoroughly cleaned. The finger was reaffixed; a vacuum of 2 Torr, effected; dry ice in acetone, added to the finger; the oil bath, positioned; and the temperature, slowly raised to 140°C. Sublimation was continued at this temperature for eight hours; then the bath was removed and the cold finger allowed to warm to room temperature. The sublimate was collected and subjected to gas chromatography and mass spectroscopy. There was no evidence of methyl esters. The spectra essentially consisted of hydrocarbons with obfuscated parent peaks. Bioassay of the

methylene chloride extract showed an average feeding response of 240 bites.

46. Re-extraction of methylene chloride extracted skin

Thirty grams of the methylene chloride Soxhlet extracted lypholyzed skin (vide supra) were continuously re-extracted with methylene chloride and methanol (50:50) for 24 hours. The solution was placed into a freezer for 24 hours, at which time it was removed and the precipitate, vacuum filtered. The filtrate was flask evaporated until a small volume of the solution remained. This solution was decanted into a sublimator and the solvent, evaporated by a stream of nitrogen gas. The sublimate was collected and subjected to gas chromatography and mass spectroscopy. The resulting spectra showed no evidence of methyl esters. Bioassay as in experiment 25 of the re-extracted lypholyzed skin indicated an average biting frequency of 220. Bioassay of the combined extract and re-extract of the lypholyzed skin indicated an average biting frequency of 250.

47. Synthesis of phenyl acetate<sup>133</sup>

A beaker, equipped with a magnetic stirring bar, was charged with 6.0 grams (0.14M) of sodium hydroxide

and 25 milliliters of water. After the sodium hydroxide had dissolved and cooled to room temperature, 10.0 grams (0.11M) of phenol were added. The solution stirred for 10 minutes. At the end of this time, 50 grams of ice were added. As the mixture continued to stir, 10.2 grams (0.10M) of acetic anhydride was rapidly poured into the beaker. After having stirred for an additional 10 minutes, the contents of the beaker were decanted into a separatory flask and extracted with 50 milliliters of diethyl ether. The organic layer was then separated and the water layer was washed with an additional 50 milliliters of diethyl ether. After the organic layers were combined and dried over  $\text{MgSO}_4$ , they were flash evaporated. The remaining oil was vacuum distilled (2mm) and a fraction collected at 44-46°C. The fraction weighed 12.9 grams, corresponding to a yield of 95% (vide spectra at end of this section). The phenyl acetate was redistilled (2mm) and a cut was collected at 44-45°C. In order to rid the sample of any trace of phenol, the phenyl acetate was column chromatographed. Bioassay of phenyl acetate evinced an average feeding response of 56 bites.

48. Synthesis of phenyl propionate<sup>134</sup>

The above procedure was repeated in synthesizing phenyl propionate. Phenol, 18.8 grams (0.20M) and 25.0 grams (0.19M) of propionic anhydride yielded 26 grams of phenyl propionate which was vacuum distilled (2 Torr) at 56-58°C (vide spectra at the end of this section). The phenyl propionate was re-distilled and a cut was collected at 56°C. Bioassay of four milligrams of phenyl propionate resulted in an average feeding response of 31 bites.

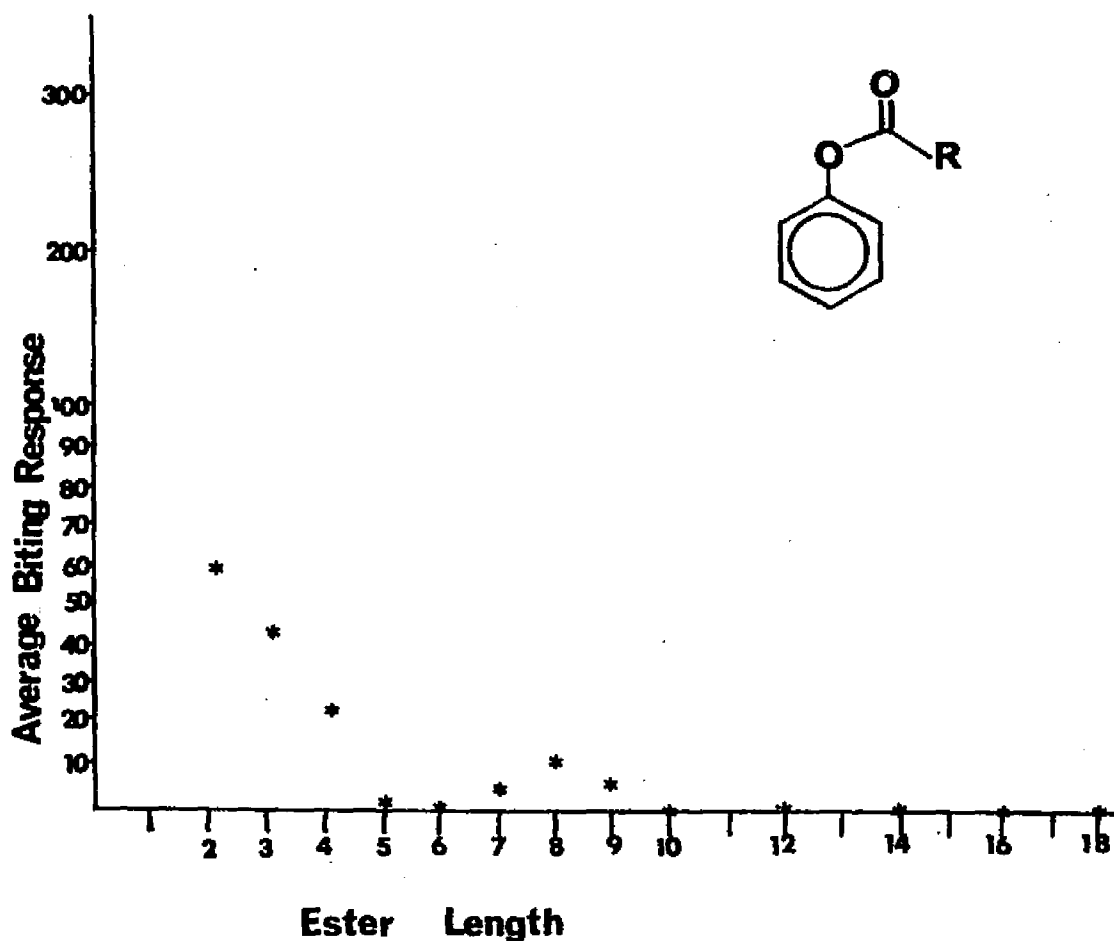
49. Synthesis of phenyl butanoate<sup>135</sup>

Into a three-necked round bottom flask, equipped with a pressure equalized dropping funnel, a magnetic stirring bar, and a West condenser containing a drying tube, was added 100 milliliters of benzene. The round bottom was charged with 8.8 grams (0.1M) of butanoic acid. Over a period of one-half hour, 16.7 grams (0.15M) of thionyl chloride was dripped into the reaction solution. The reaction was then allowed to stir for 21 hours; whereupon heat was applied until reflux was obtained. The reflux was continued for an additional two hours. Subsequently, the reaction was cooled to room temperature. Next, 9.4 grams (0.1M) of phenol together with 2 milliliters of pyridine were added. This solution stirred



six more hours. The contents were then poured into a separatory flask and extracted twice with 100 milliliter portions of saturated sodium carbonate. The organic layer was collected and dried over  $\text{MgSO}_4$ ; subsequently, the benzene was removed by flash evaporation. The resulting oil was vacuum distilled (2mm) and a fraction was collected at  $74-76^\circ\text{C}$ . The clear oil weighed 11.2 grams, a weight which corresponded to a 68% yield (vide spectra at the end of this section). Bioassay of phenyl butanoate evinced an average feeding response of 20 bites.

This procedure was used to synthesize the following compounds (vide end of this section for their spectra): phenyl hexanoate,<sup>136</sup> phenyl heptanoate,<sup>137</sup> phenyl octanoate,<sup>138</sup> phenyl nonanoate,<sup>139</sup> phenyl decanoate,<sup>140</sup> phenyl dodecanoate,<sup>138</sup> phenyl tetradecanoate,<sup>138</sup> phenyl hexadecanoate,<sup>138</sup> and phenyl octadecanoate.<sup>138</sup> The bioassay of these various compounds is indicated in the Graph 2.



Graph 2

50. Synthesis of ethyl 2-hydroxydecanoate

A three-necked round bottom flask, equipped with a magnetic stirring bar, was fitted with a condenser, an equalized pressure dropping funnel, and a Claisen head. The flask was charged with 13 grams (0.2M) of newly sandpaper polished zinc ribbon and 300 milliliters of dry benzene. The benzene was distilled until approximately 100 milliliters remained. While

heating was continued, the Claisen head was quickly replaced by a ground glass nitrogen inlet and positive nitrogen pressure was maintained throughout the course of the reaction. An admixture of 16.7 grams (0.1M) of ethyl bromoacetate and 12.8 grams (0.1M) of octanal in 50 milliliters of dry benzene was poured into the dropping funnel. Ten milliliters of the admixture was rapidly added to the refluxing benzene. After 15 minutes, refluxing was stopped and one crystal of iodine, inserted; five minutes later, two chips of freshly crushed magnesium were added. No initiation was observed. After an additional five minutes, five drops of 1,2-dibromoethane was dripped into the mixture; ebullition was noted. The solution was again brought to reflux; the zinc metal turned grayish, indicating commencement of the reaction. The remainder of the bromo ester and aldehyde was added dropwise over a 35 minute period. At this time, the dropping funnel was replaced by a glass stopper. After 12 hours, an additional 10.5 grams (0.6M) of ethyl bromoacetate was poured into the reaction vessel. The reaction was refluxed for six more hours; whereupon heating was terminated and the contents of the flask, cooled to 0°C. Then, 50 milliliters of 6N hydrochloric acid were

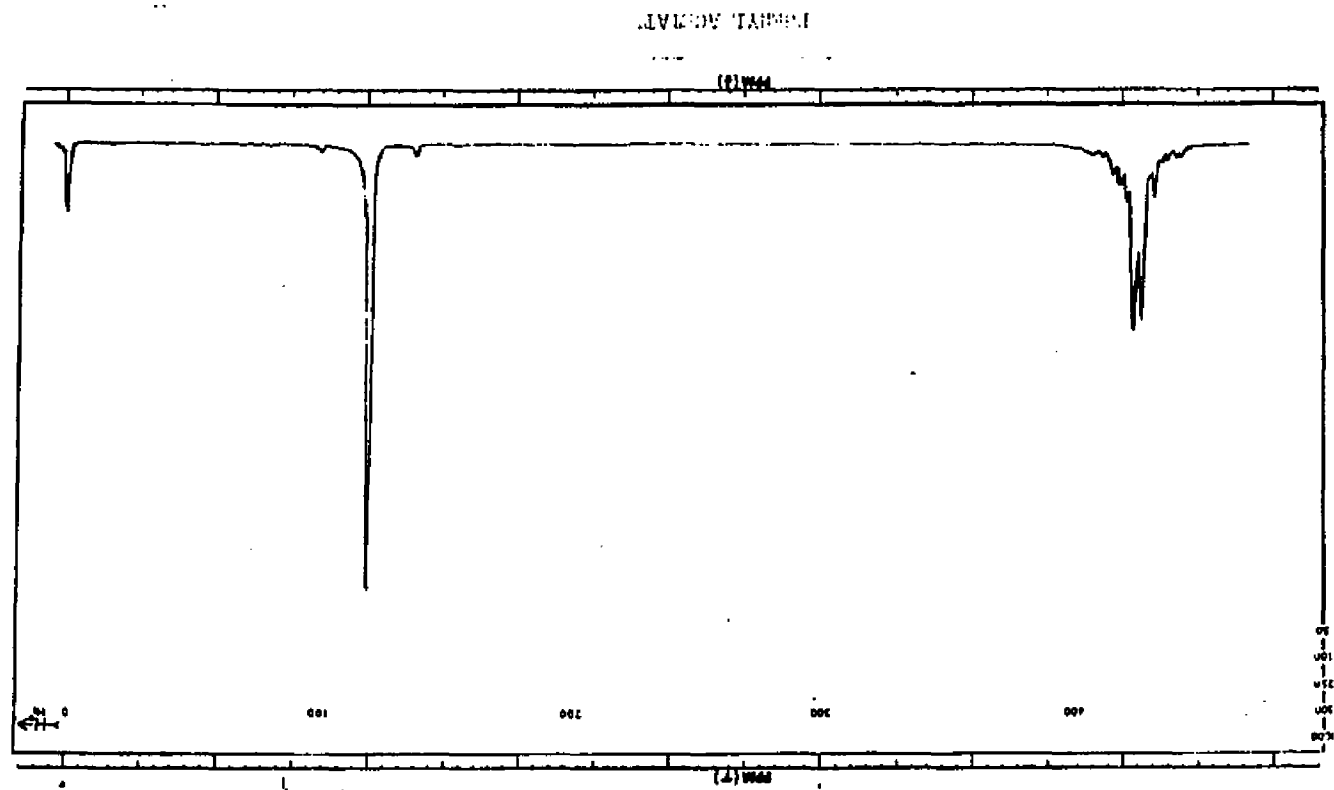
inserted and the reaction mixture was stirred for one hour. At the end of this time, the contents of the round bottom flask was poured into a separatory flask, and the organic layer was collected. The organic layer was subsequently washed with 100 milliliters of brine water and 100 milliliters of water. It was then dried over  $\text{MgSO}_4$  and the solvent was flash evaporated. The residue was vacuum distilled. Three fractions were collected: one at  $70-95^\circ\text{C}$ ; another at  $120-123^\circ\text{C}$ ; the third at  $135-145^\circ\text{C}$ . The fraction collected at  $120-123^\circ\text{C}$  was redistilled. The resulting clear oil weighed 7.6 grams, a weight which corresponds to a 35% yield (vide spectra at the end of this section). Bioassay of this material proved negative. Bioassay of the hydrolyzed ester was also negative.

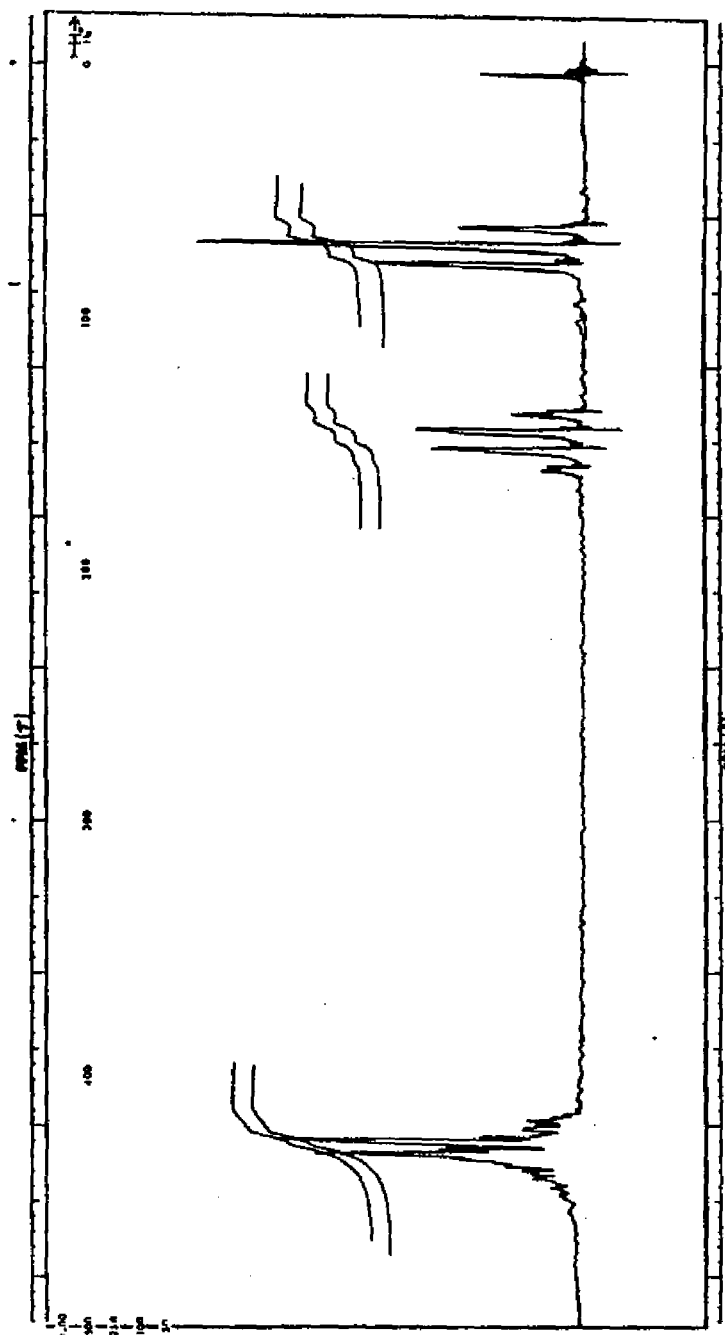
51. Chemicals tested for food attractant activity

1-octene	oleic acid
2-heptanone	iso-stearic acid
3-heptanone	amygdalic acid
2-octanone	butyric acid
2-tridecanone	heptanal
2-undecanone	decyl alcohol
isophorone	propionic acid
valeric acid	acrylic acid

tannic acid	salicylic acid
acetic acid	catechol
furoin	resorcinol
acetaldehyde	salicylaldehyde
pyrogallol	p-cresol
gallic acid	o-cresol
anisol	p-nitrophenol
phloroglucinol	methyl salicylate
coumarin	oleyl alcohol

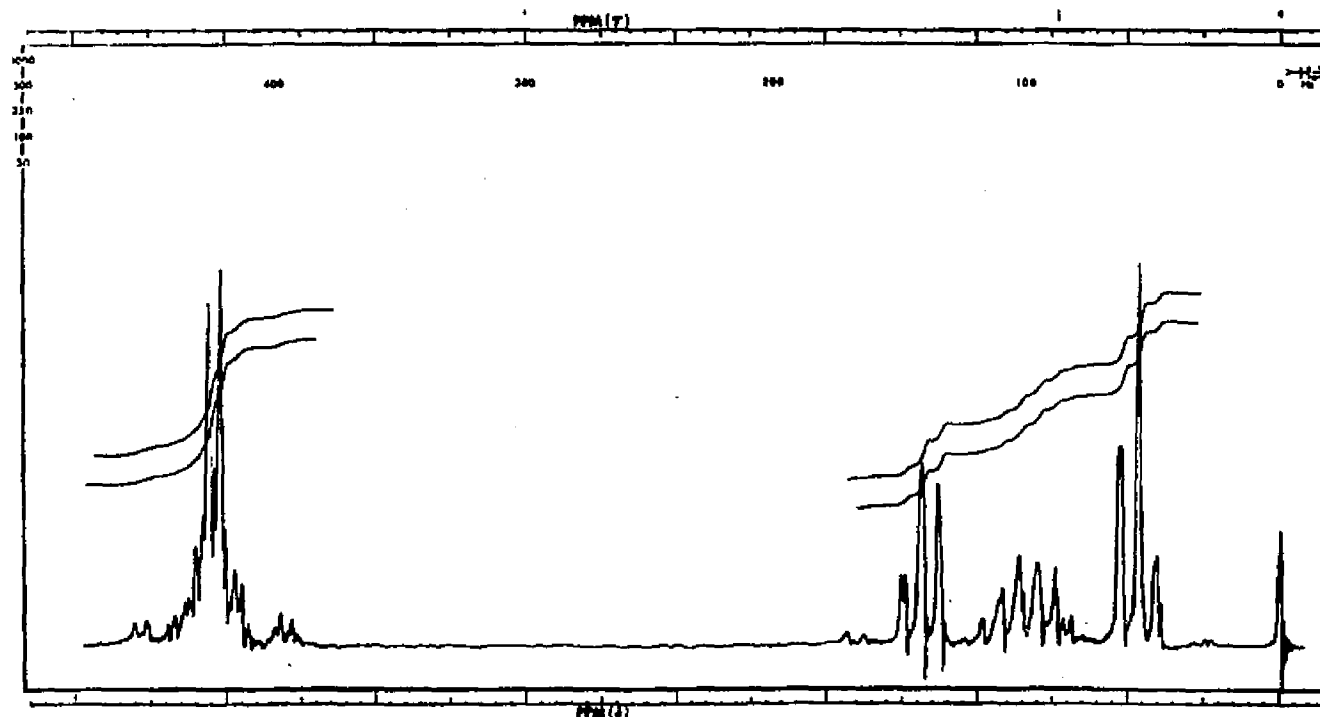
The above compounds were tested for biological activity by applying four milligrams of each on three corks and subsequently exposing them to weevils for 21-24 hours.





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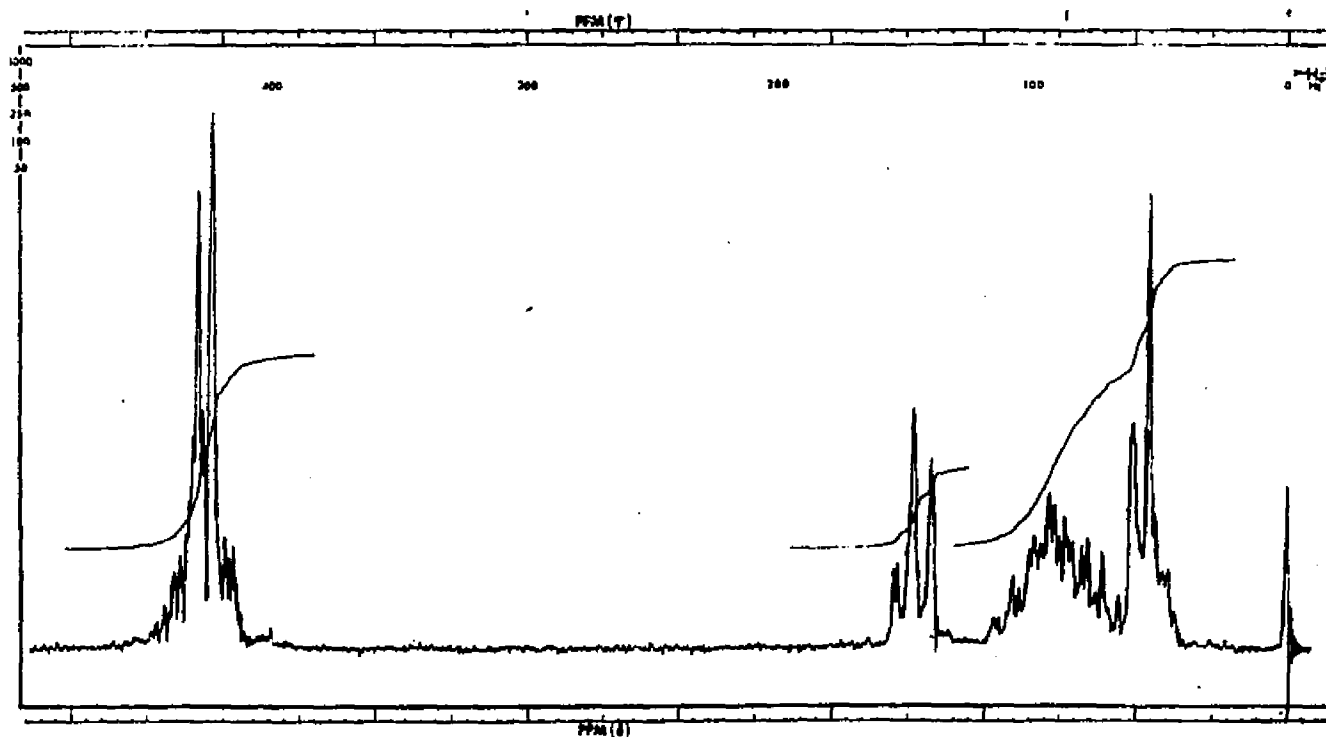
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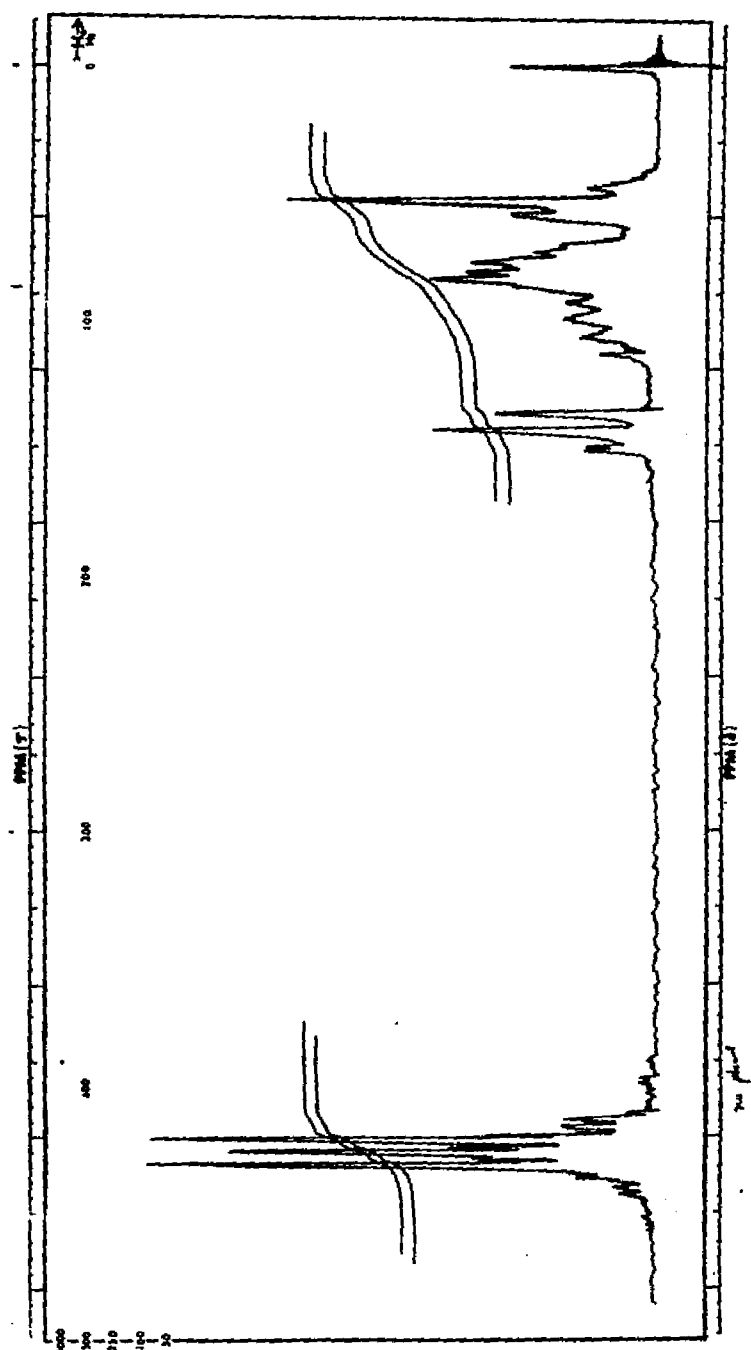
PHENYL BUTANOATE

125



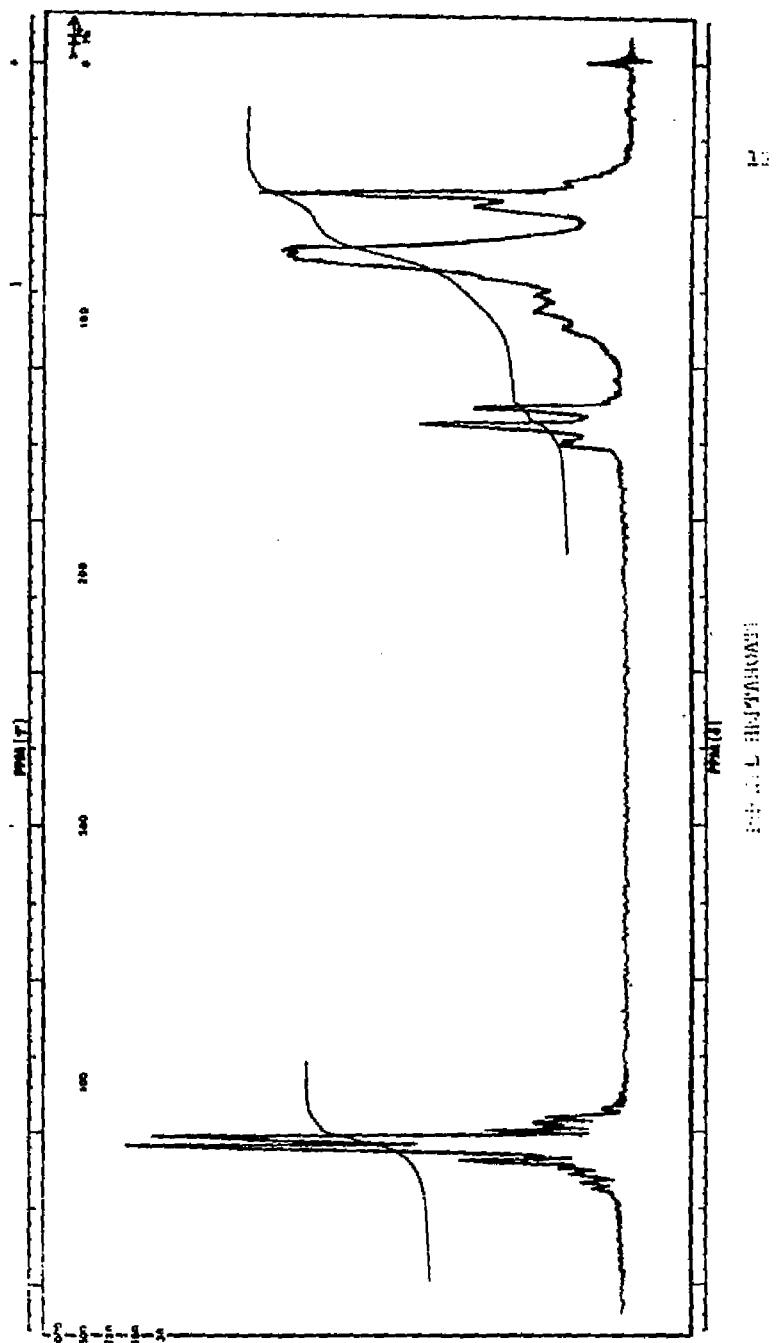


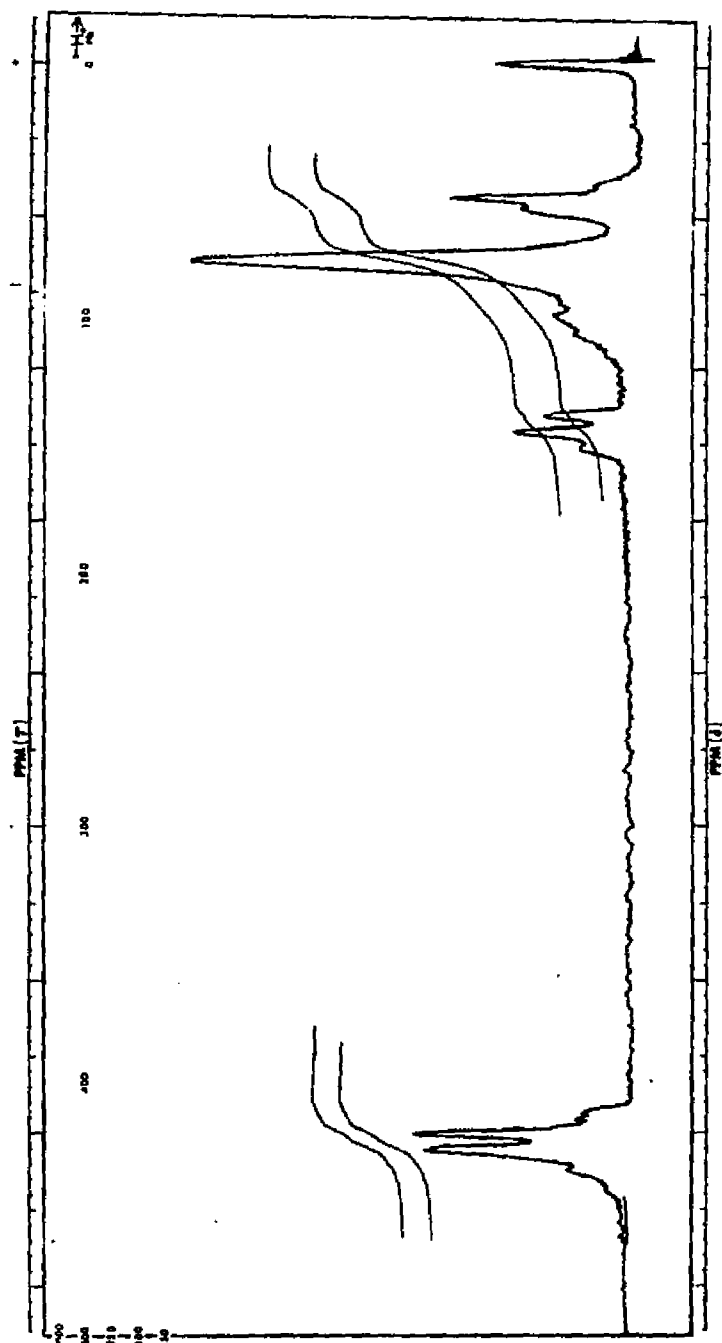
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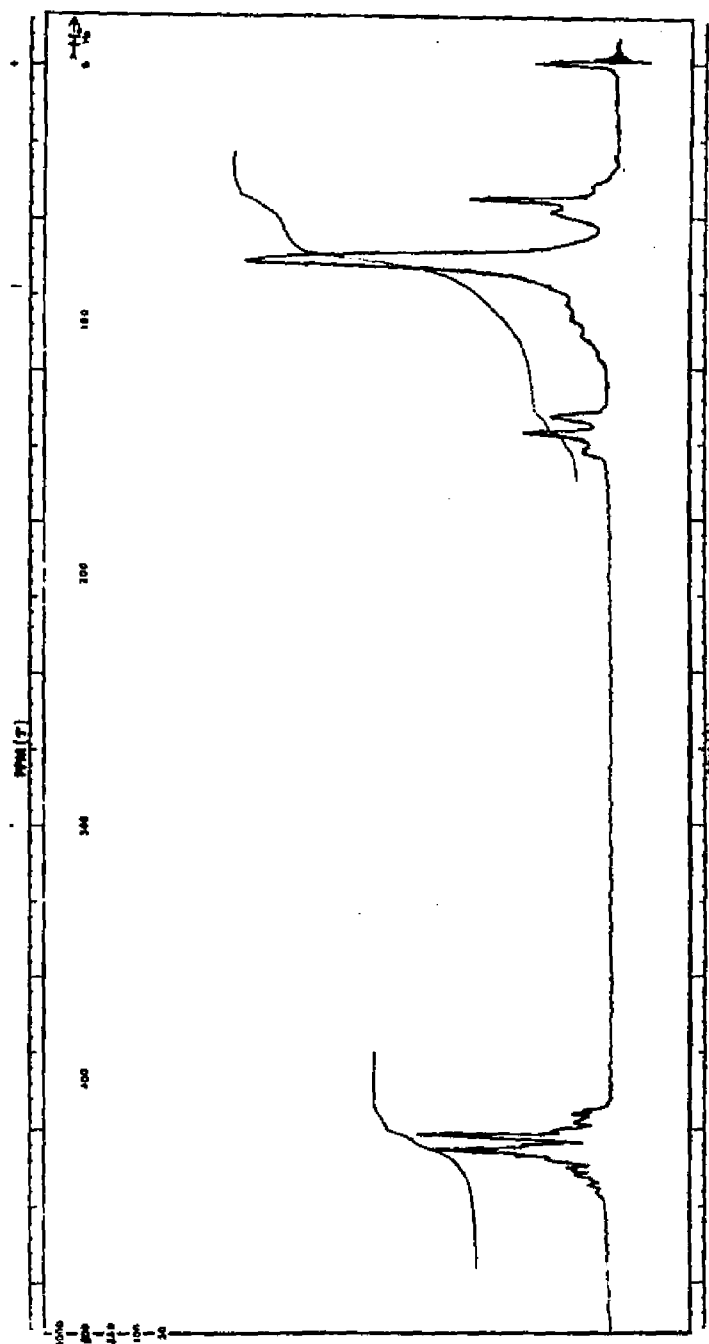
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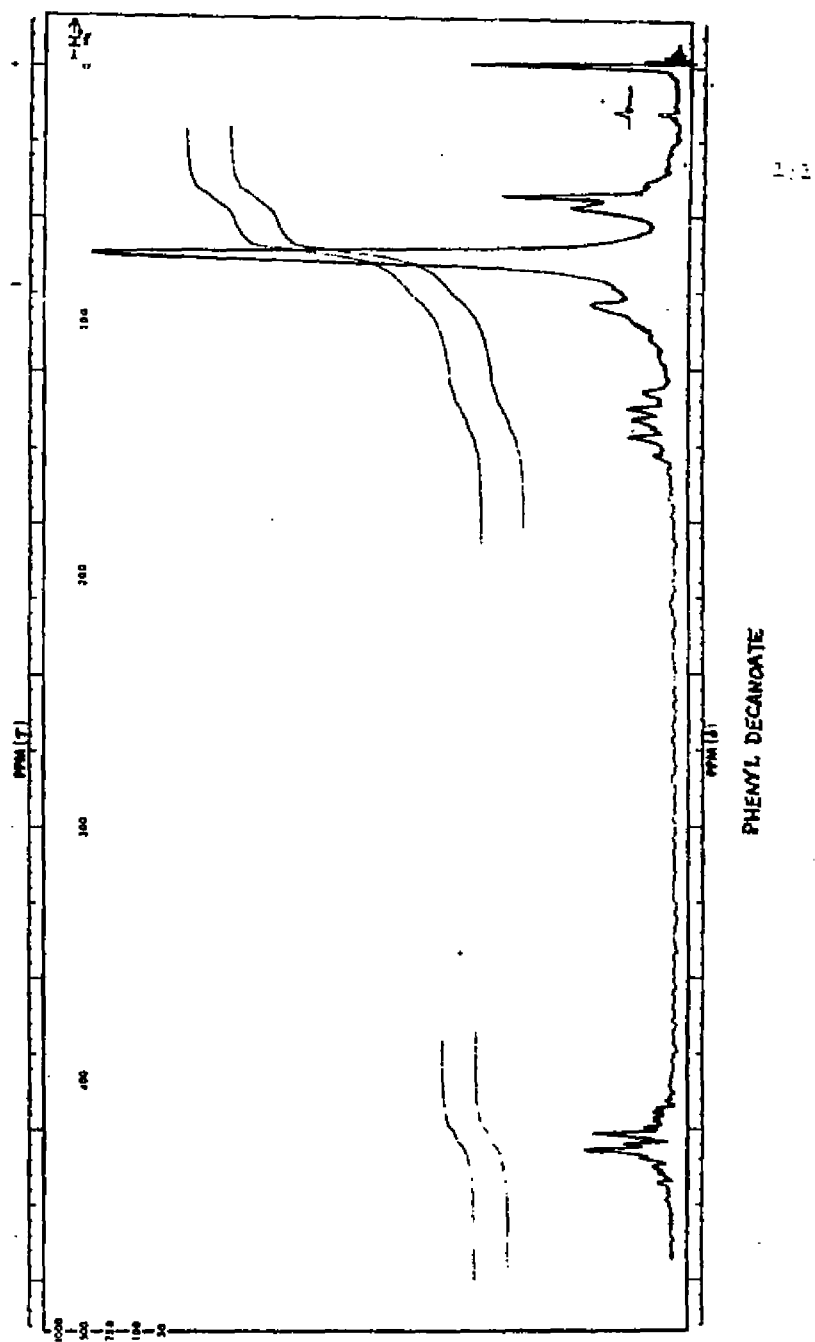


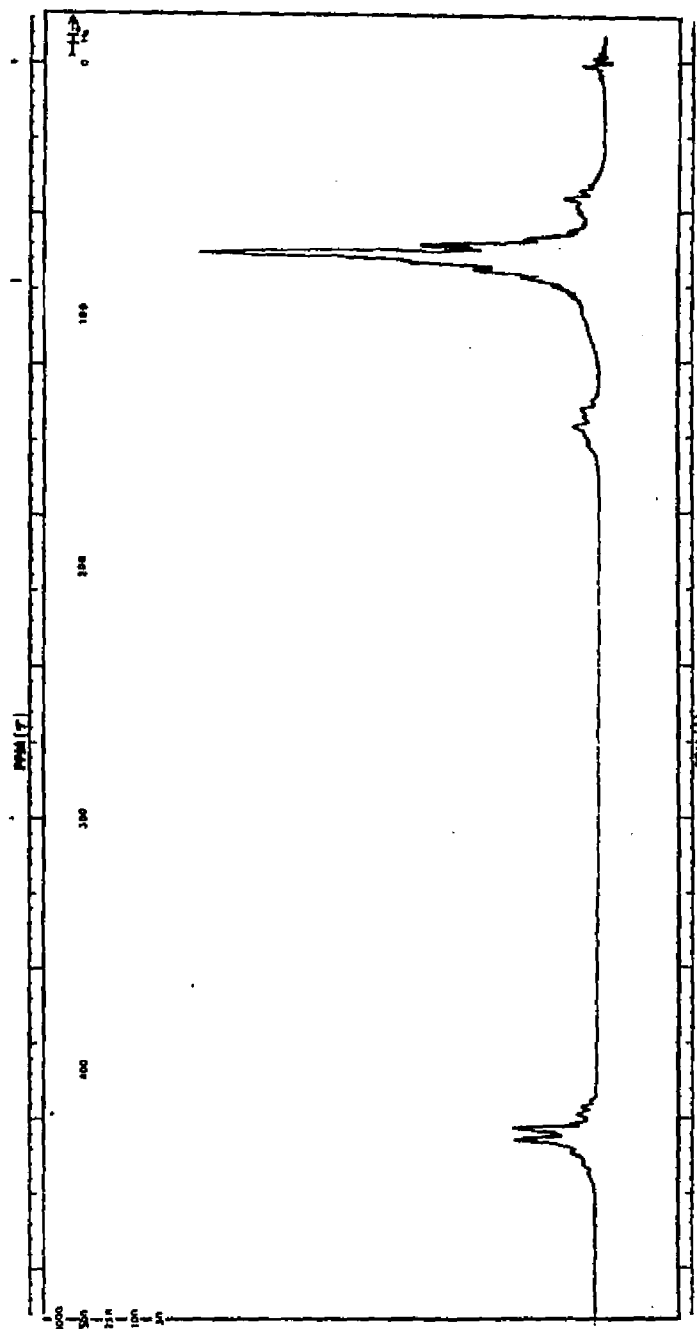


methyl acetate



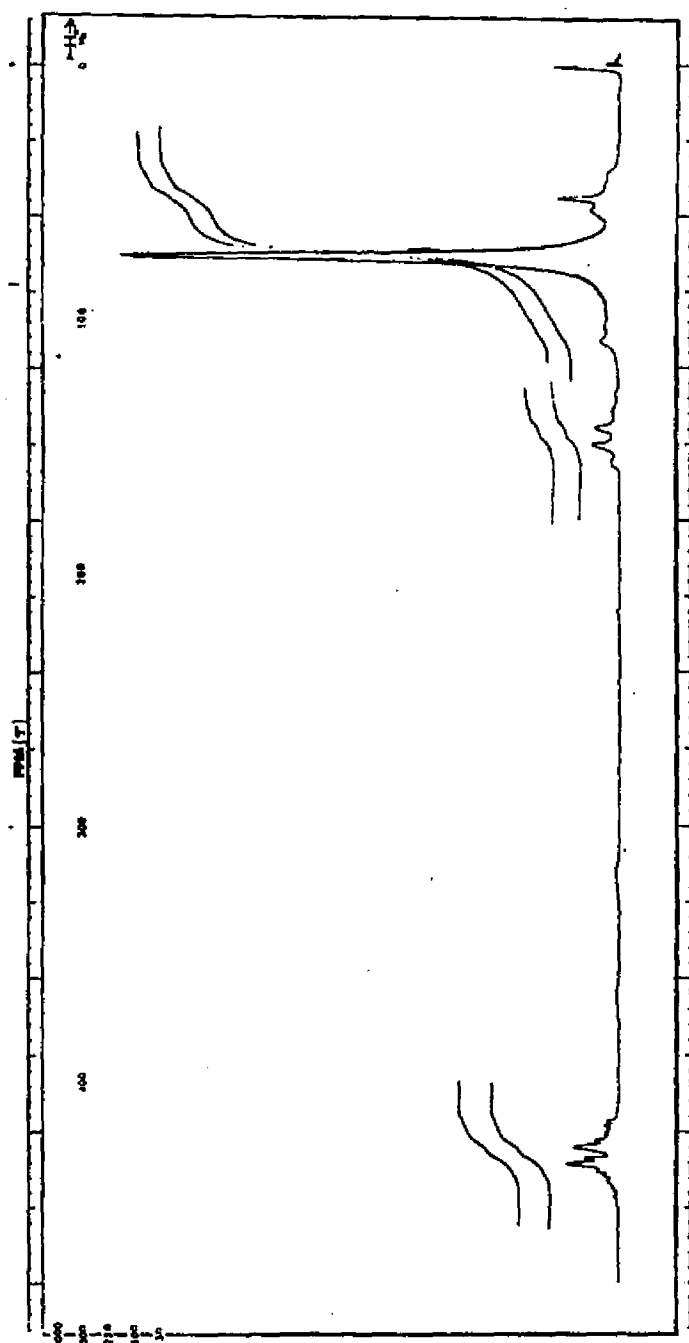
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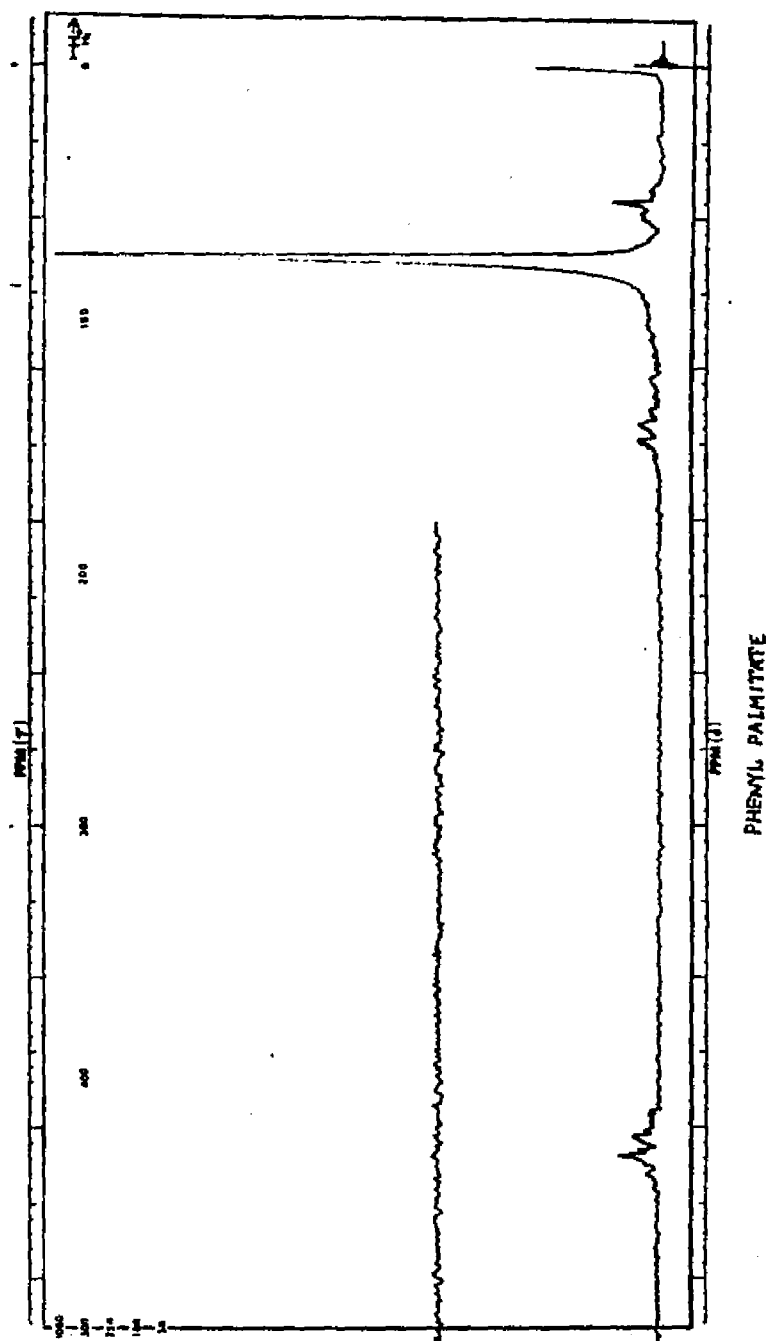
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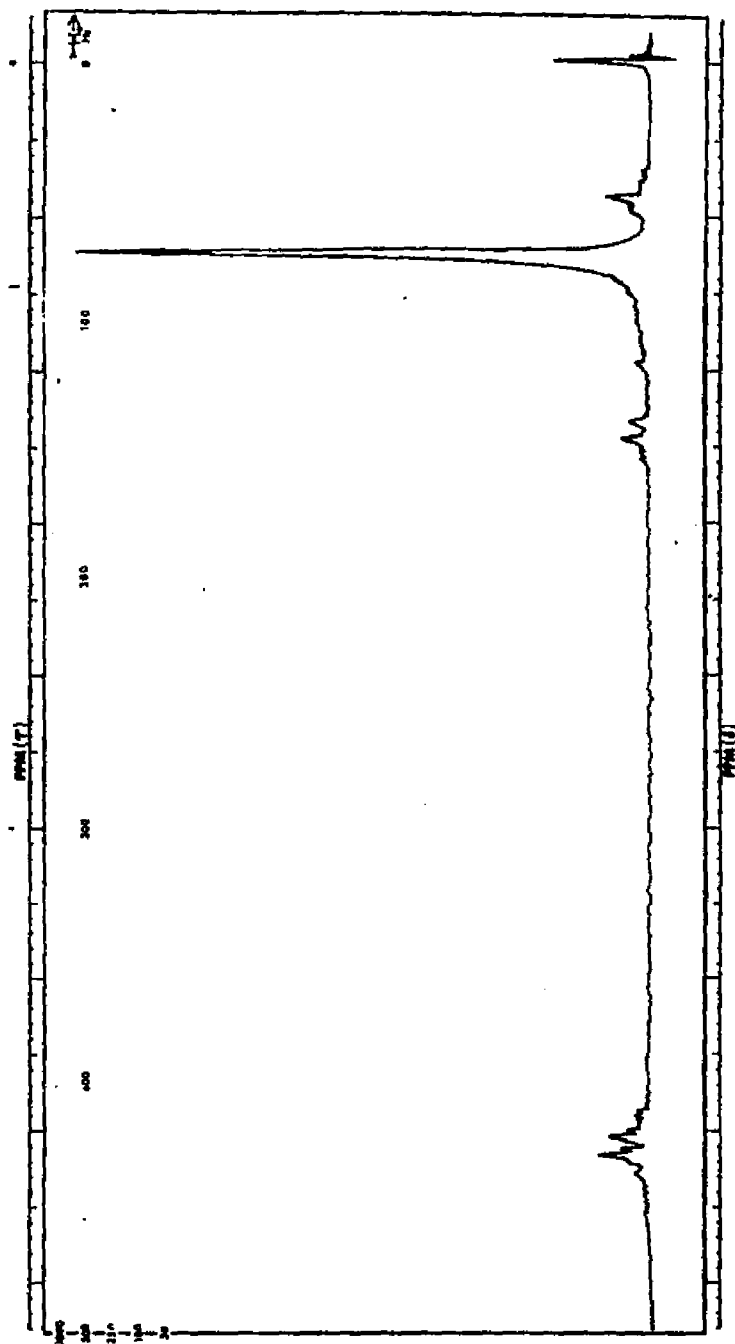
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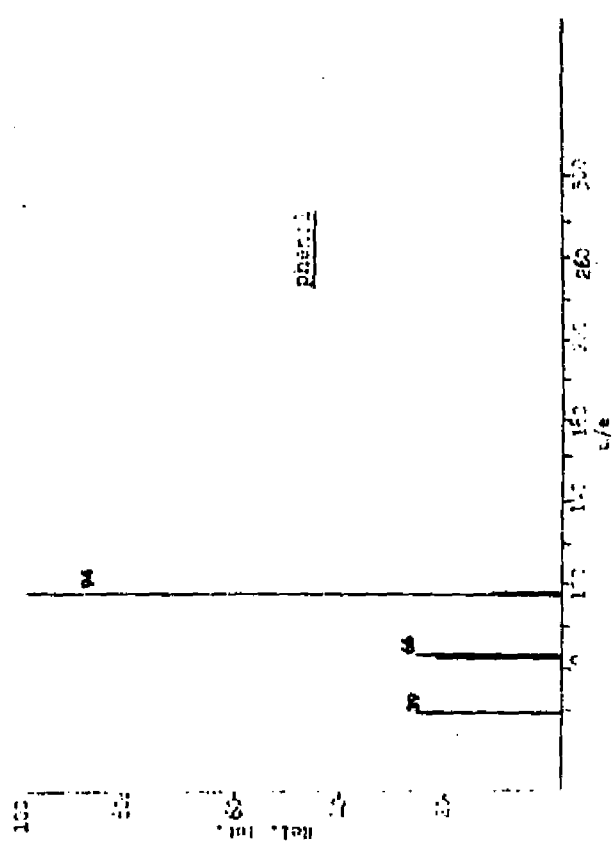
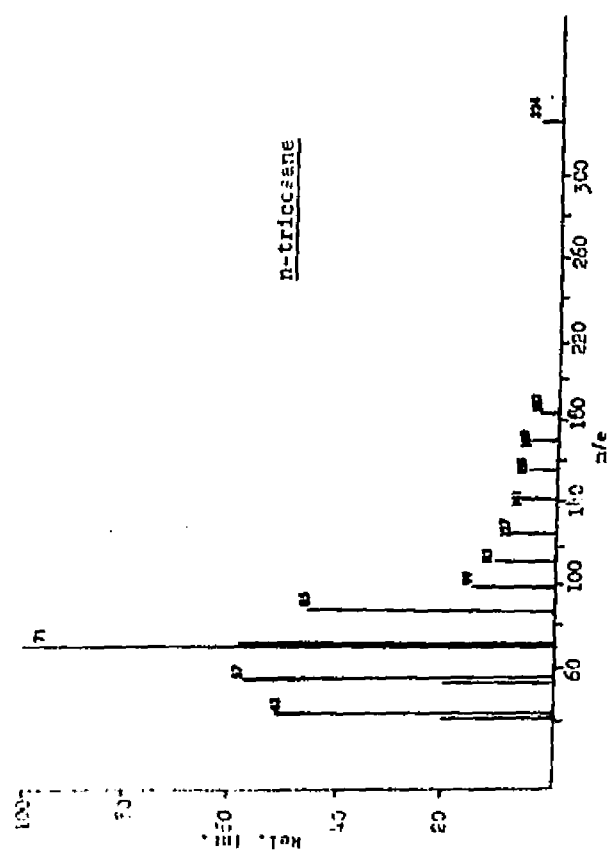
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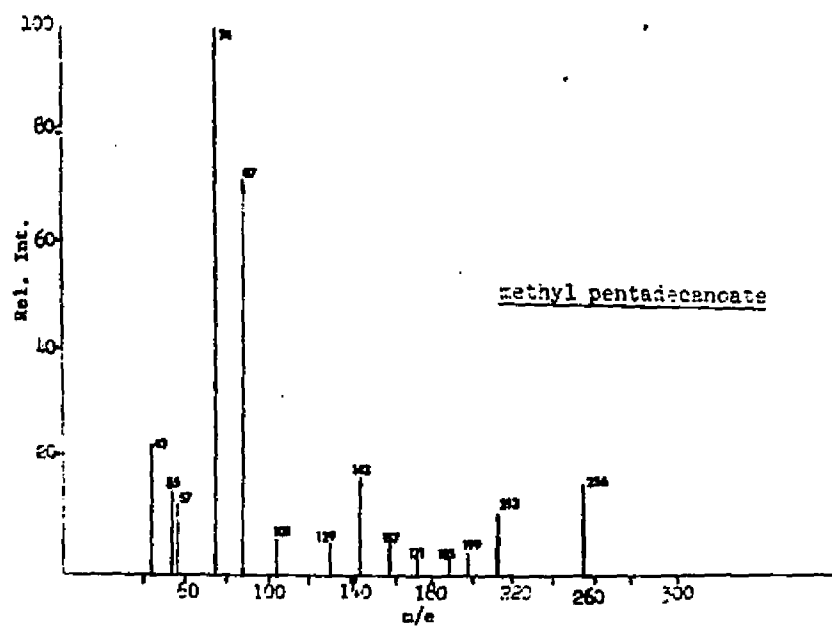
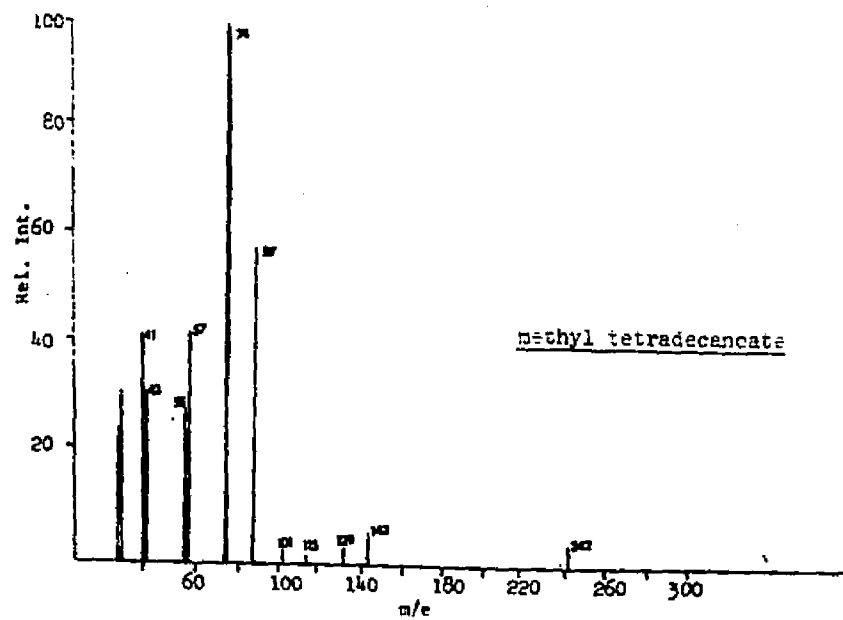


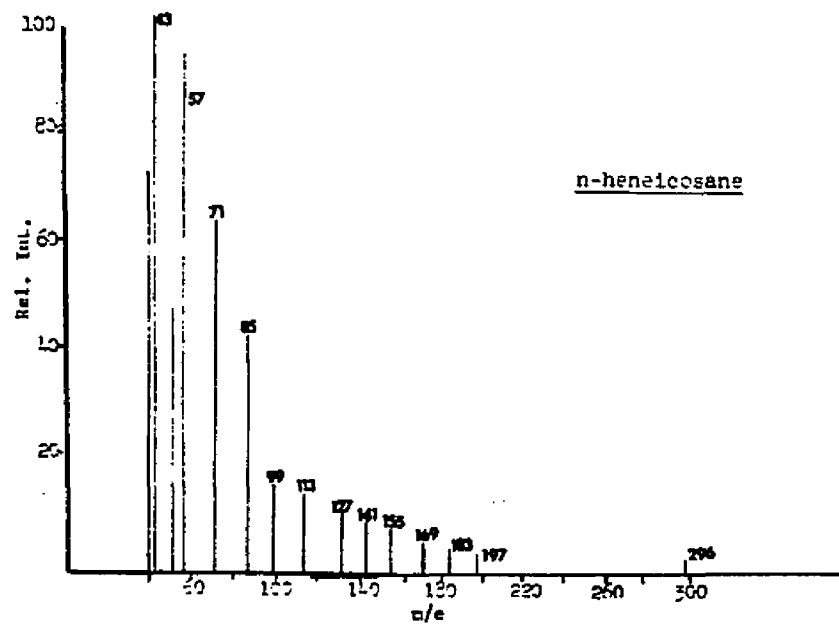
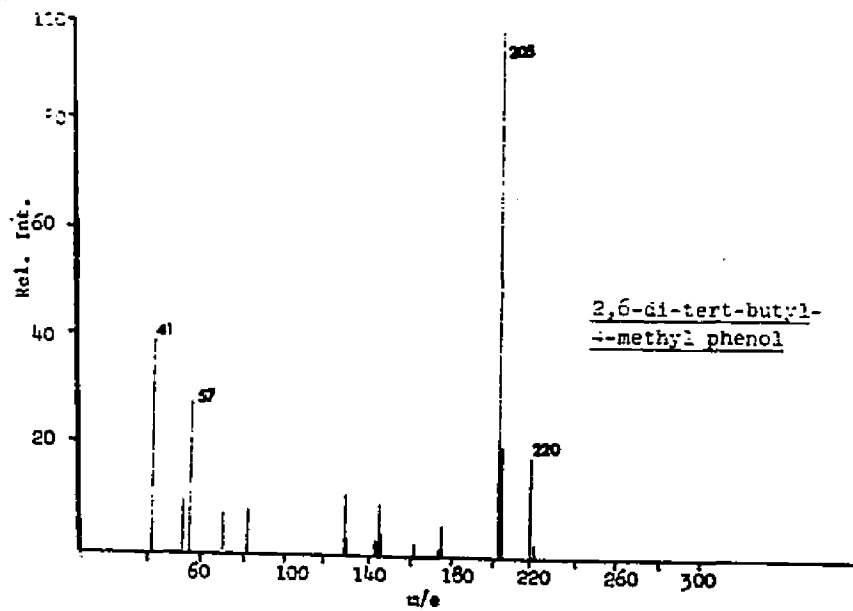


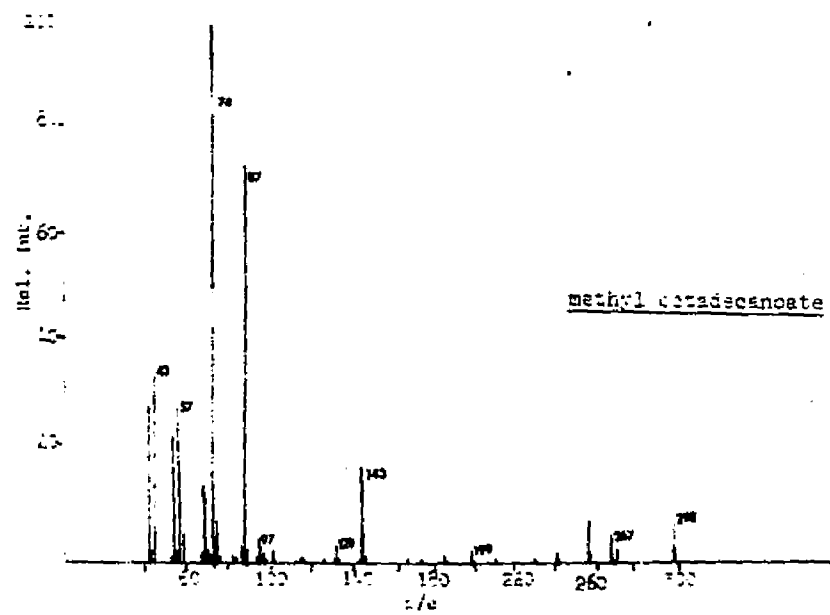
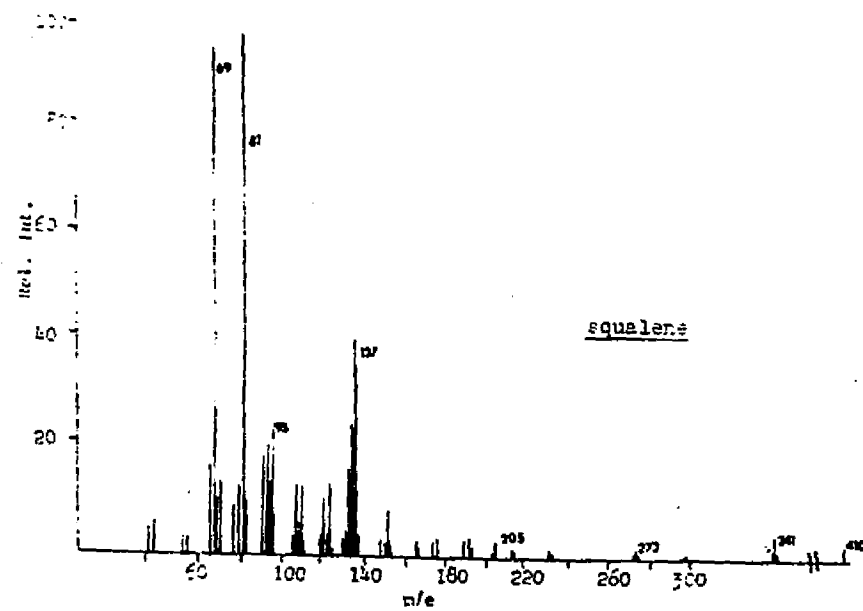


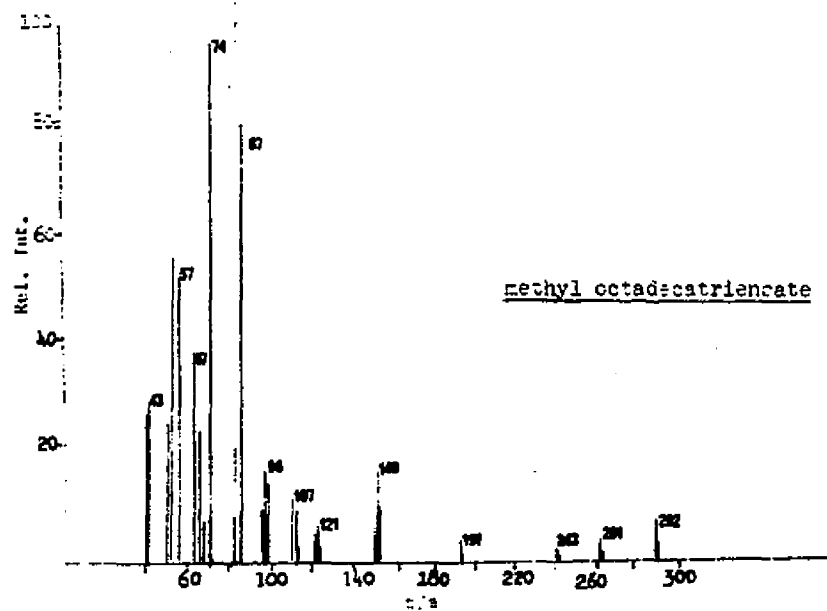
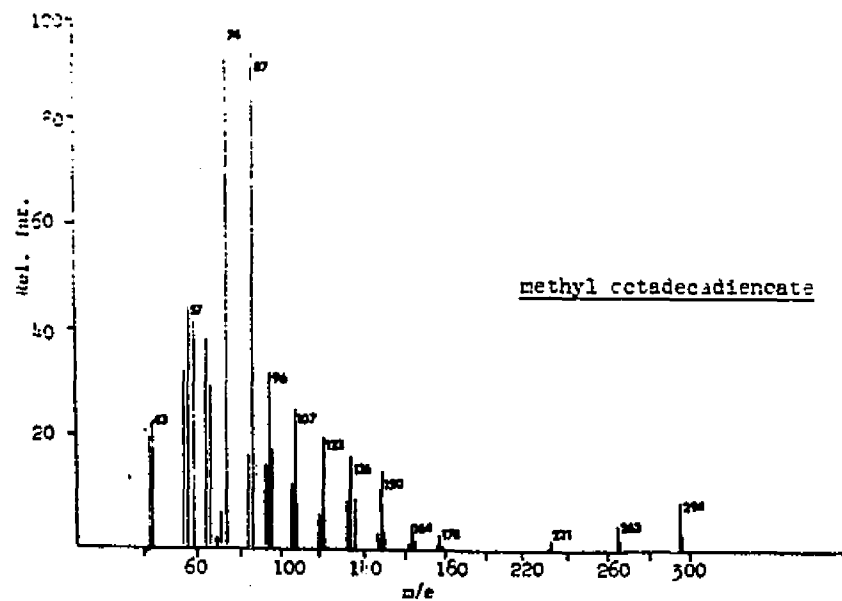
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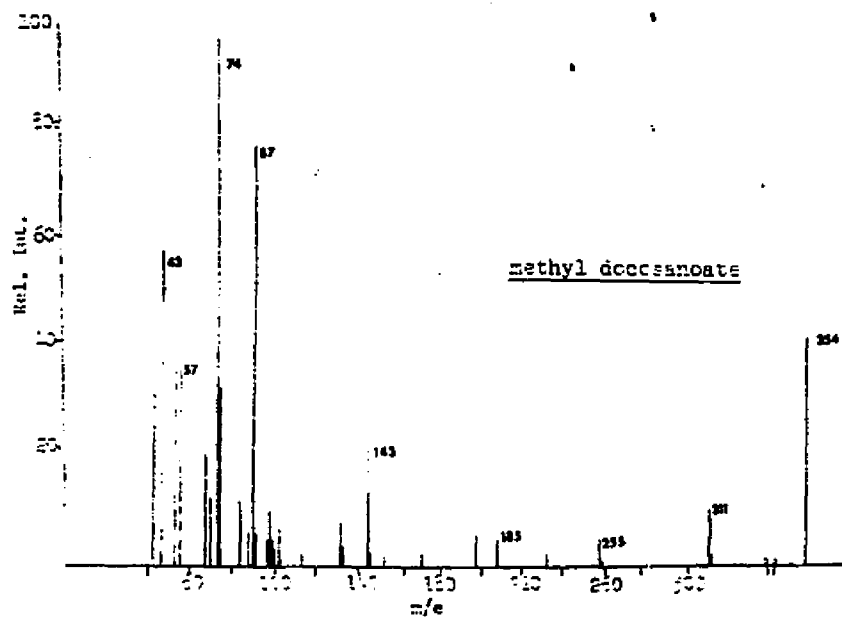
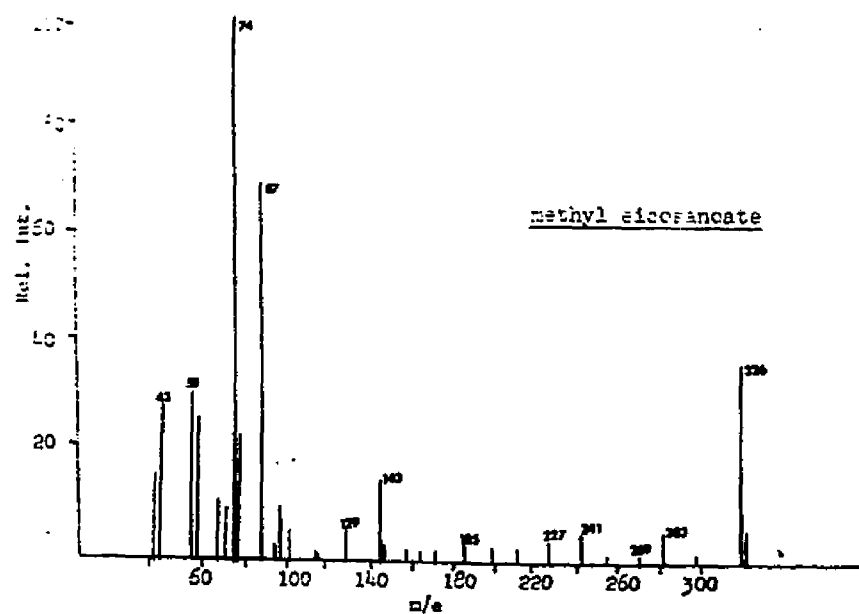




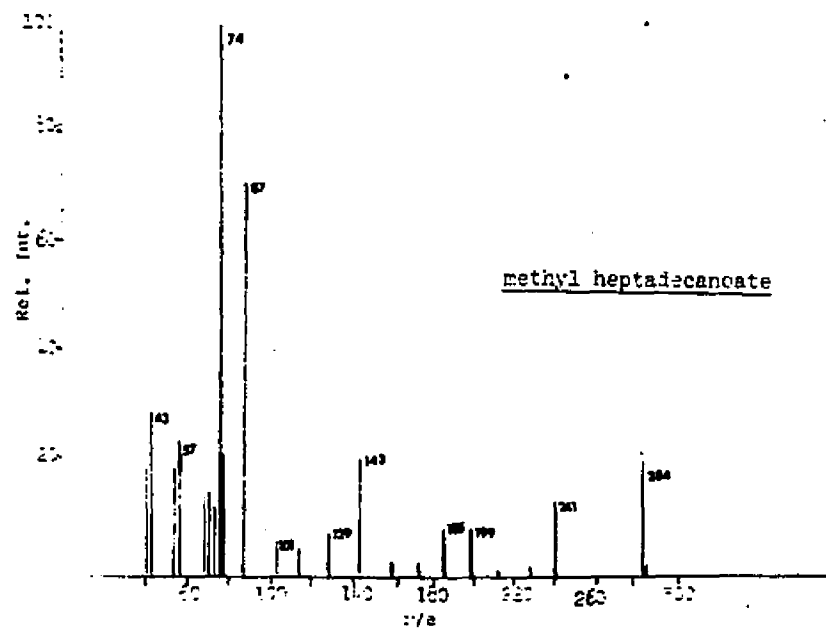
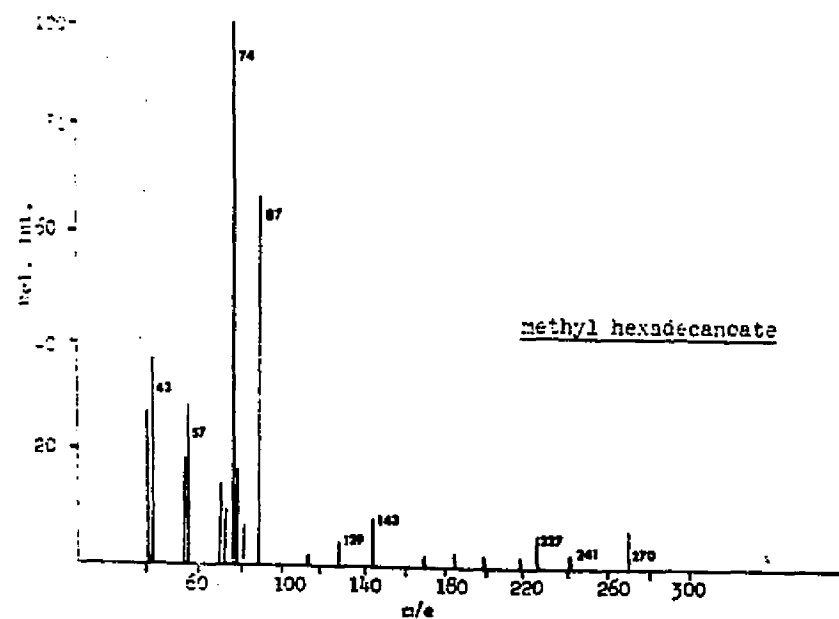






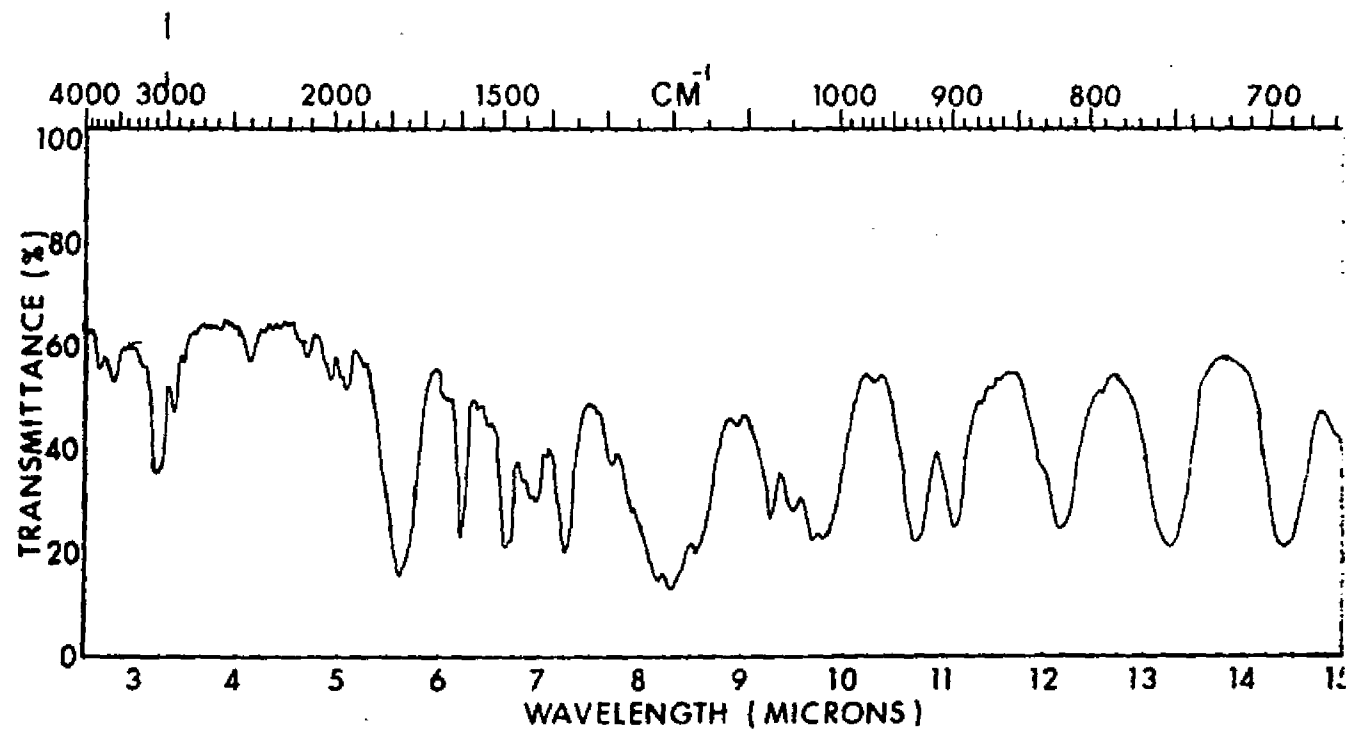




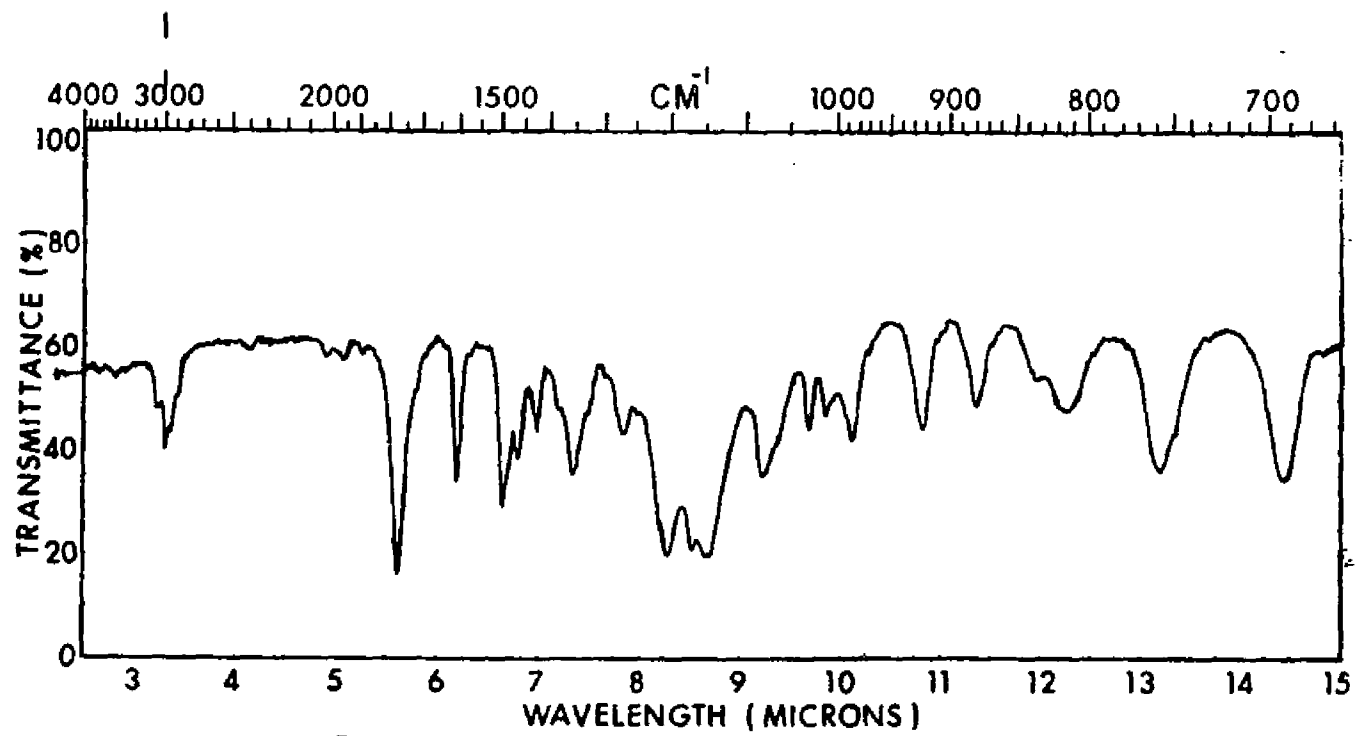


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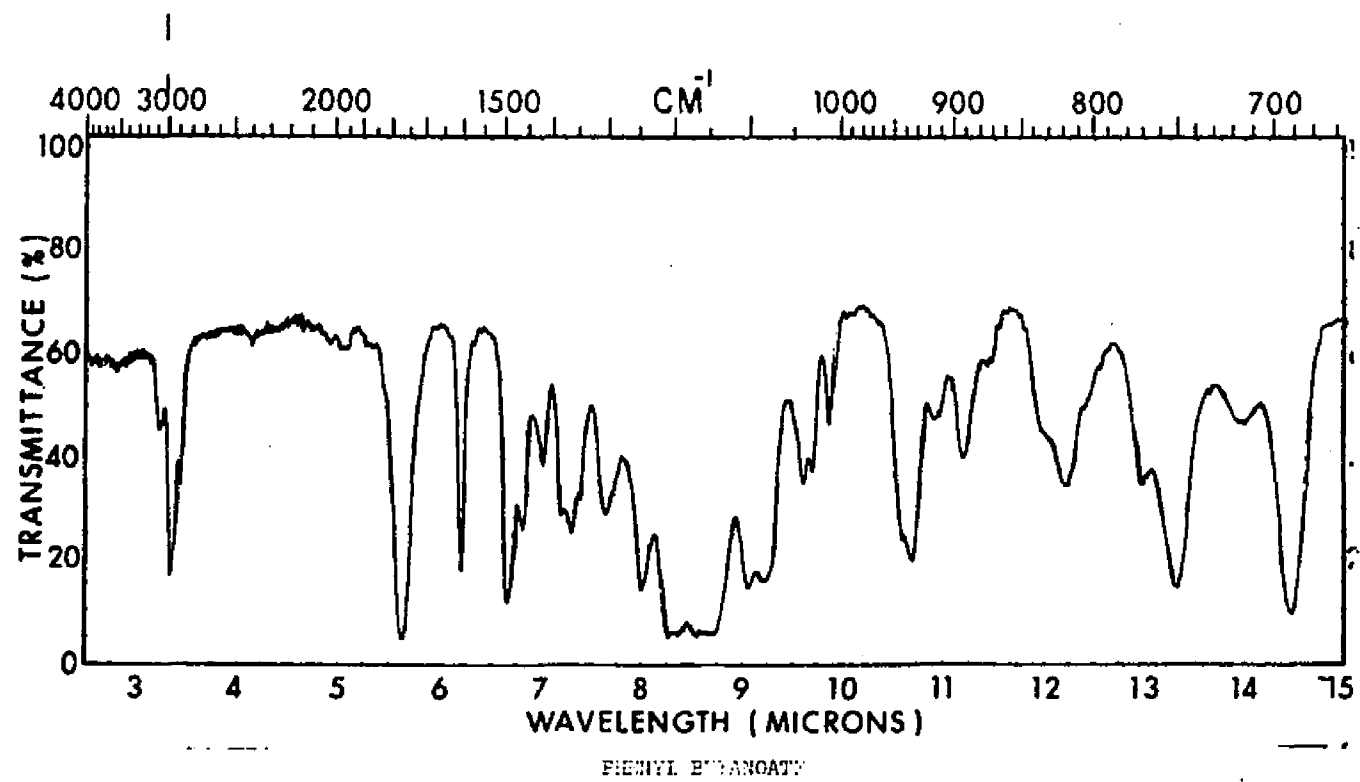


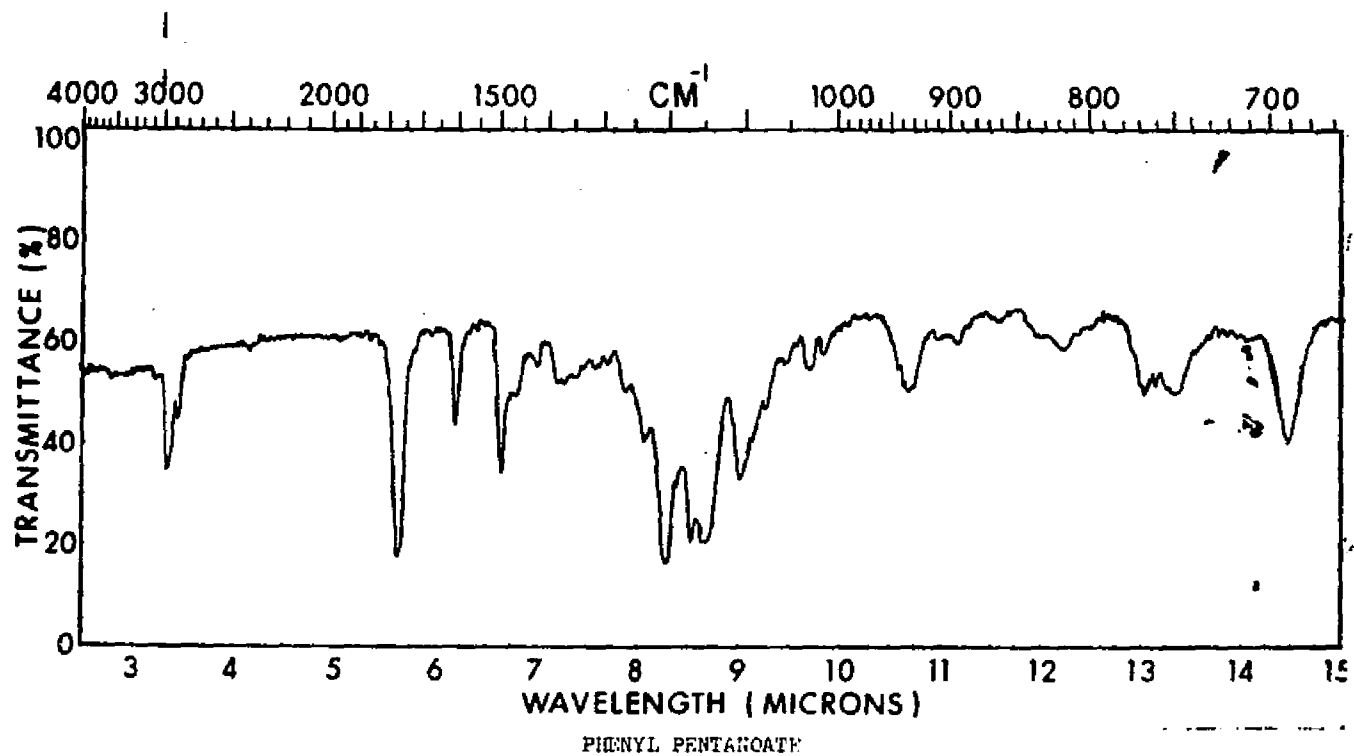
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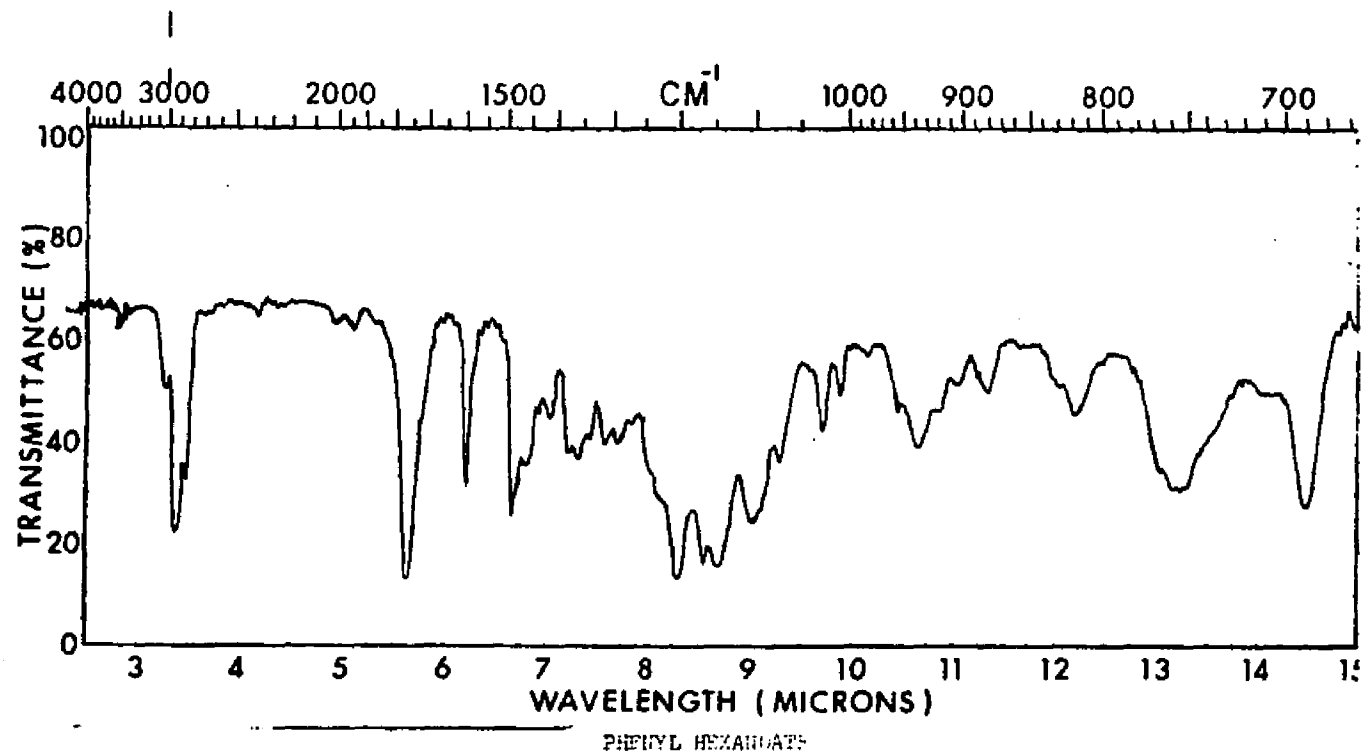


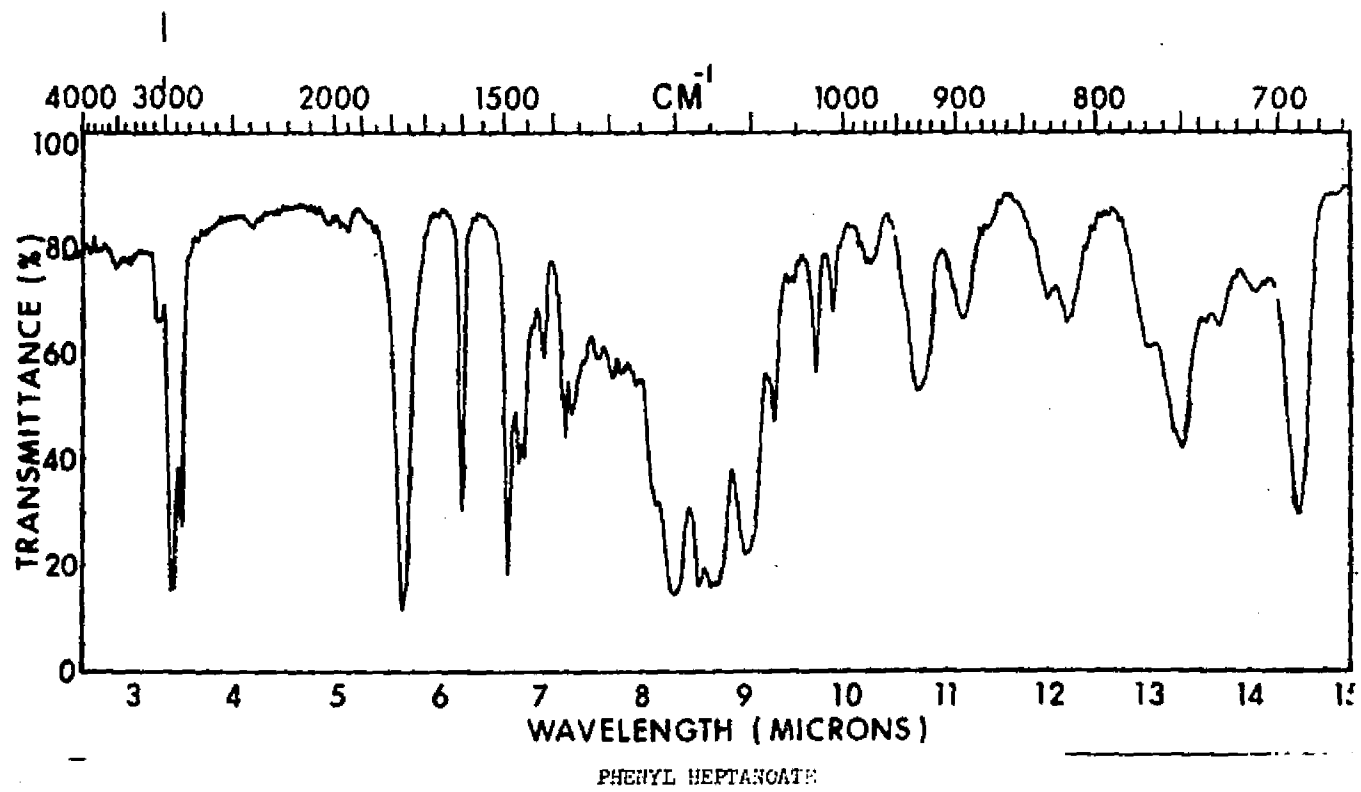
PHENYL PROPIONATE

Fig.

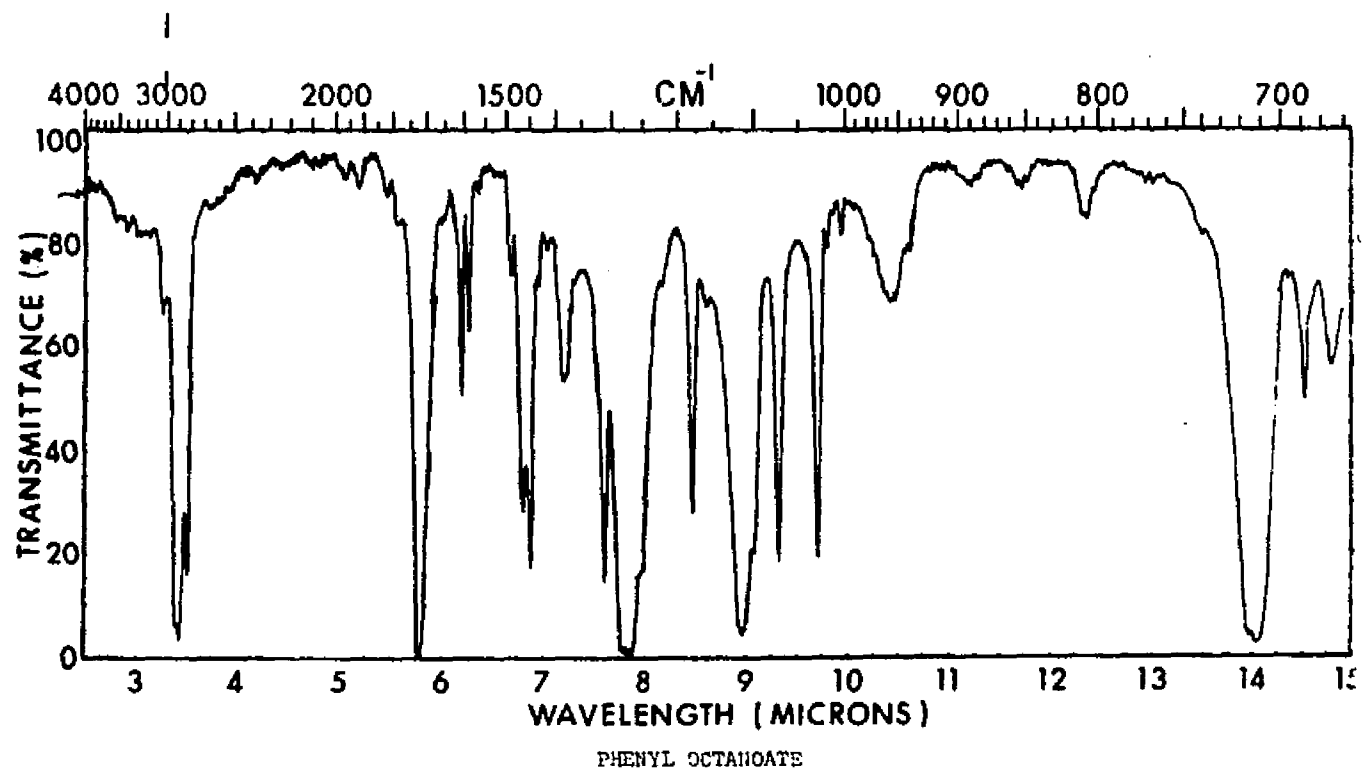


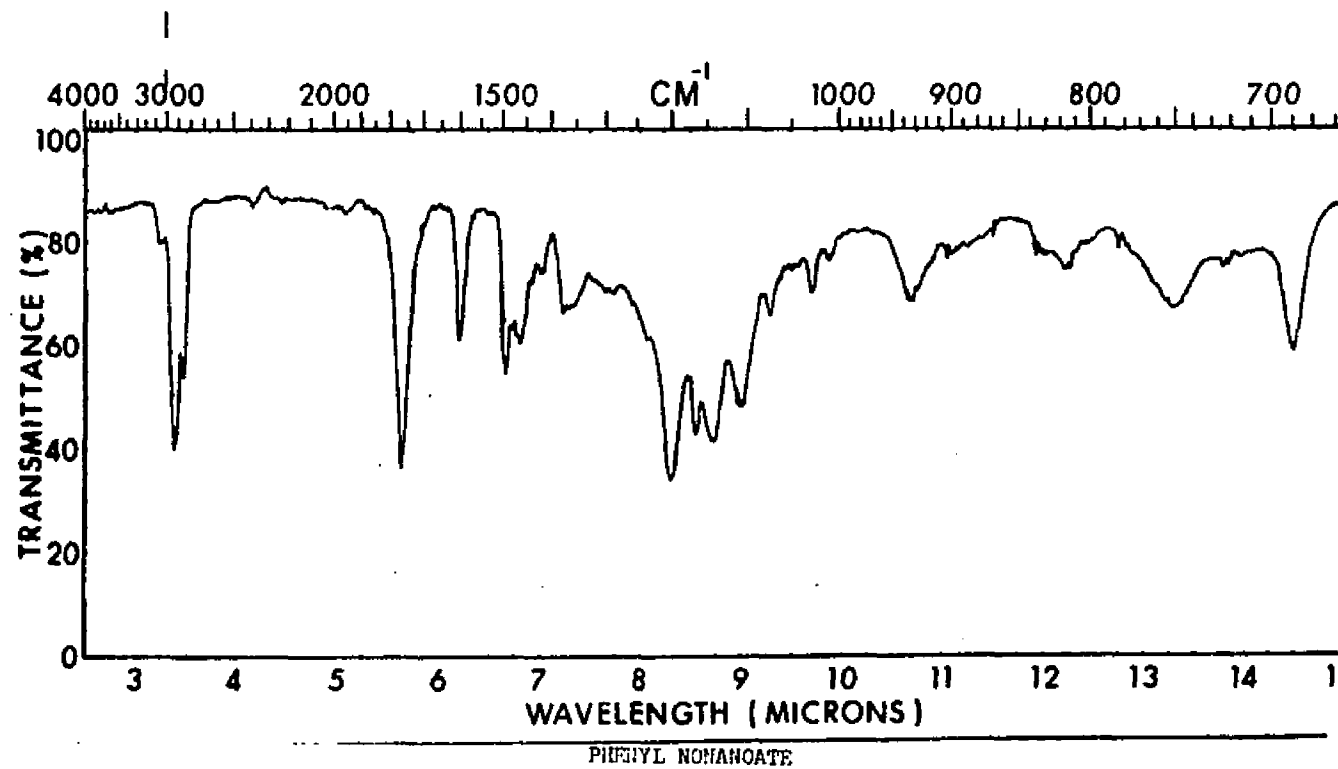






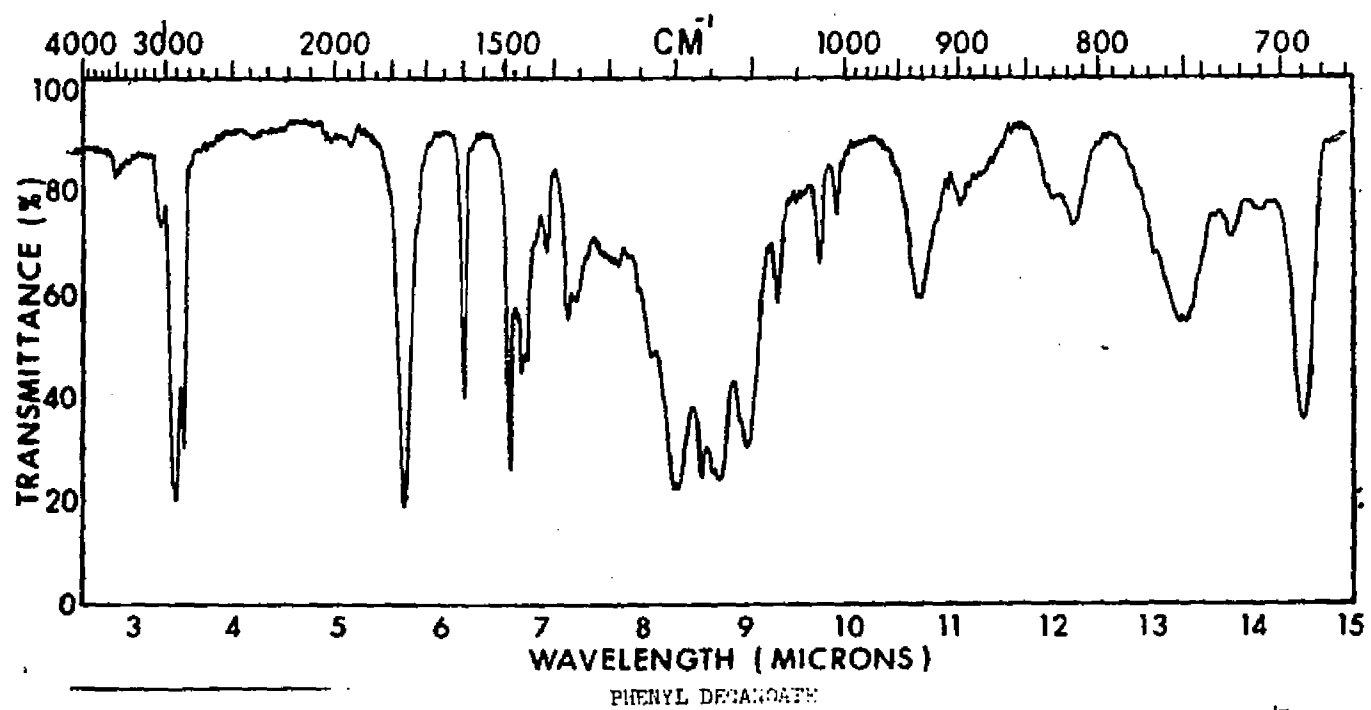


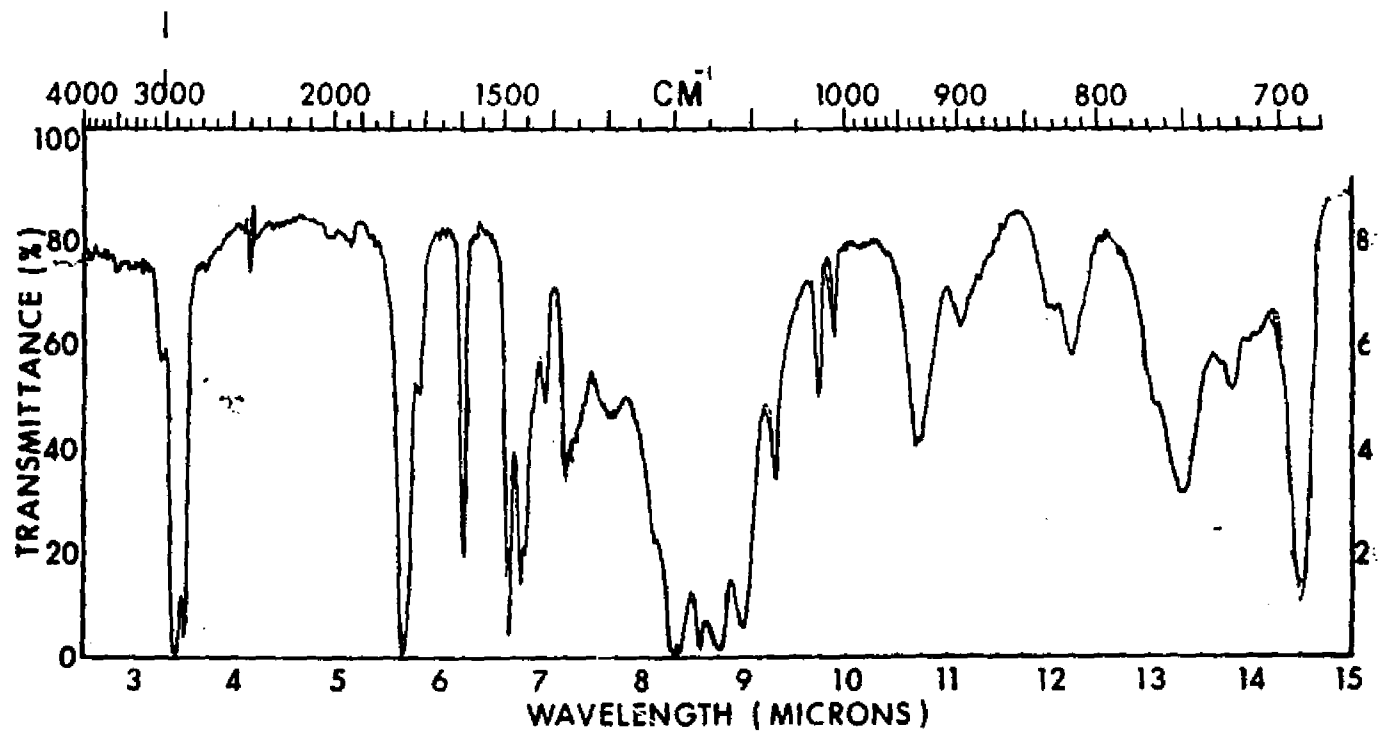




PIPERYL NONANOATE

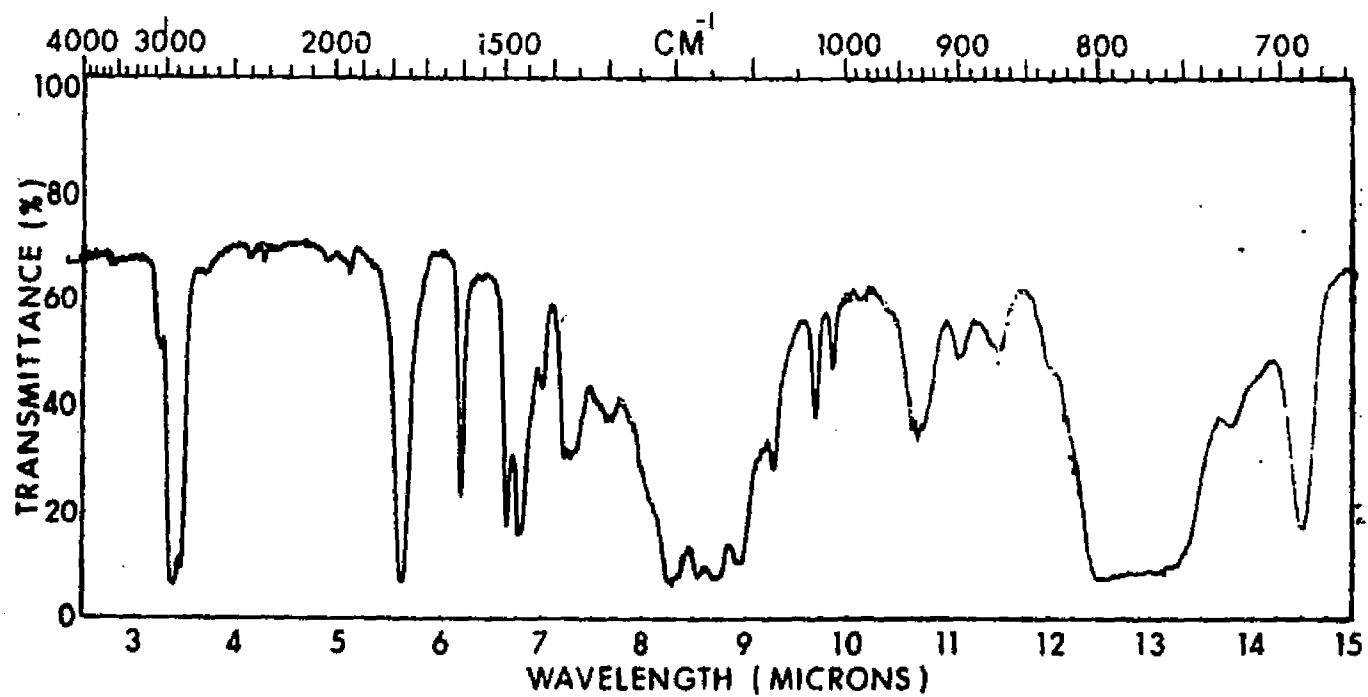
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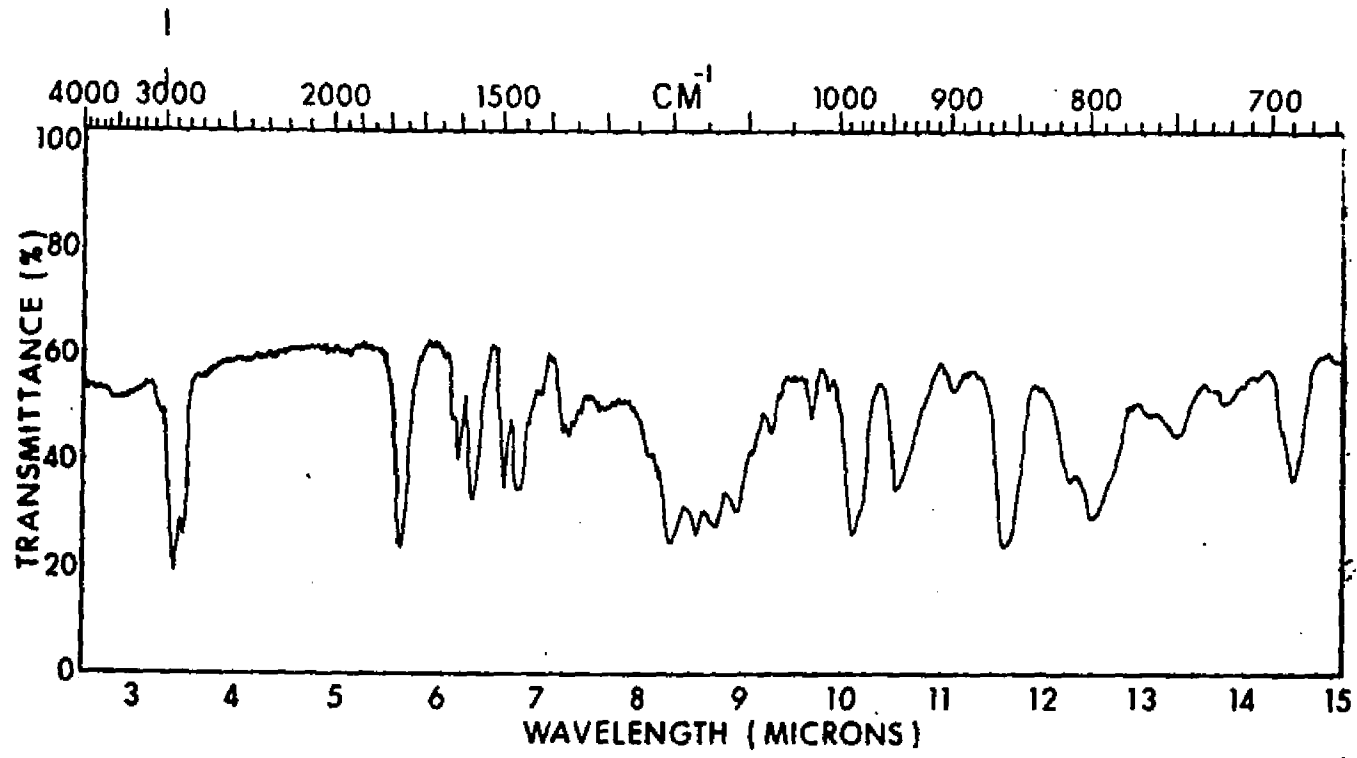


PHENYL LAURATE

153

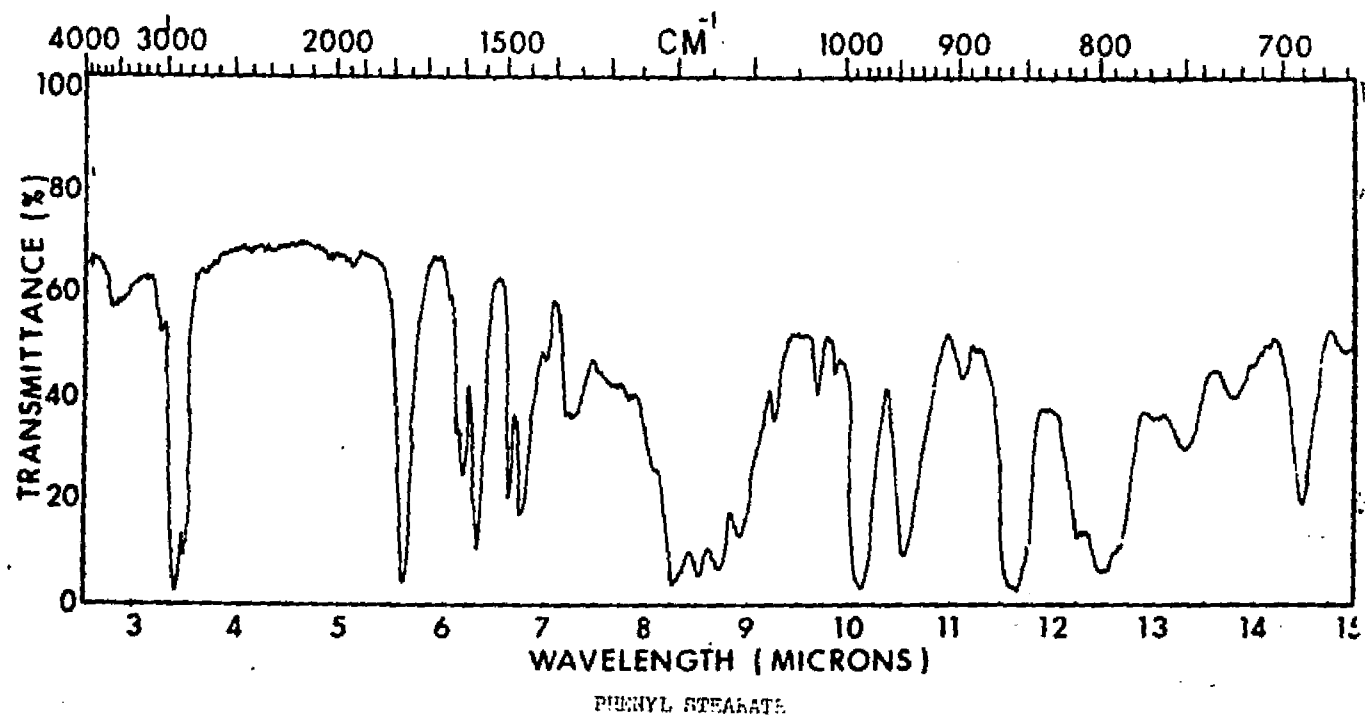


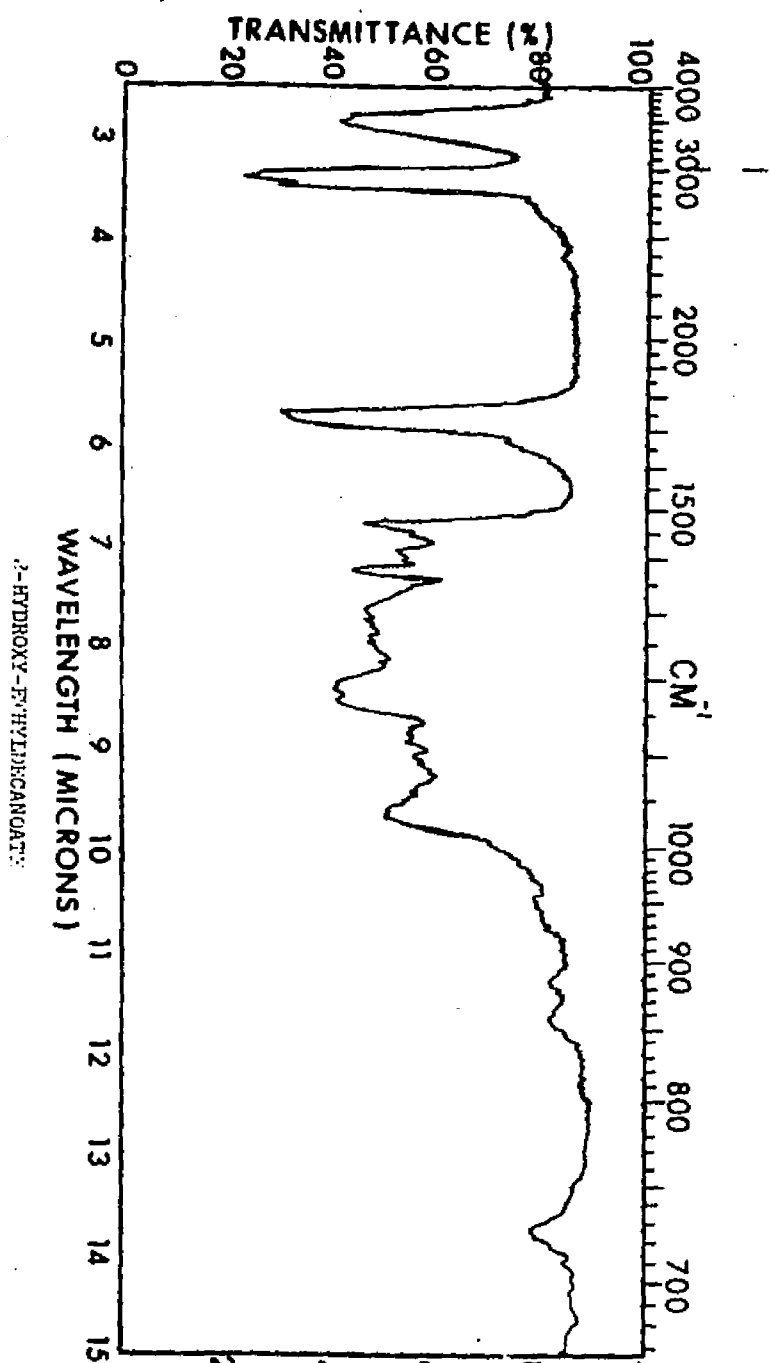
POLYETHYLENE TEREPHTHALATE



PHENYL PALMITATE

155







## CHAPTER III

### DISCUSSION

Within the last thirty years, agricultural production has increased rapidly because science has found chemical means of effectively controlling the insects which formally decimated at least half of the potential agronomic yield. The scientific notion of pesticides obviously serves a purpose: with the introduction of chemical poisons, the destruction of annual agricultural crops by pests has been significantly reduced. Additionally, when chemical progress is coupled with such basic pursuits as phytogenetics, the prognosis for the agricultural industry would seem ostensibly sound. Unfortunately, this is not the case and phytogenetics has not been the weak link. To the contrary, phytogenetic advancements have proved themselves agriculturally and ecologically beneficial. For instance, N. E. Borlaug, the Nobel laureate, has developed the technique of plant breeders to a point where a variety of wheat will give manifold yield--enough perhaps to allay an imminent famine in the Third World. The

weakness of the agricultural revolution is a basic weakness of most revolutions--over-confidence. When scientists realized the effectiveness of the chemical insecticides, initially they zealously advocated its broad-spectrum use. Farmers saw the bountiful effect of this use in their crop production. Yet despite subsequent warnings by the scientific community<sup>141</sup> against haphazard proliferation of insecticides, the farmer paid little heed. His interest was in an impressive crop yield, and he was confident that he was using the required amount of chemical poison to kill the insects which were now developing an increasing level of resistance.

The result of this proliferation has been poignant and a circular crisis now exists: the food demands of the population impose the necessity of pesticides to meet these demands; with the use of pesticides, insects develop a level of resistance;<sup>142</sup> in order to counteract this level, additional or more lethal poisons are contingent; at the same time, the population explosion demands more food. The cycle is complete. As it repeats itself, its spirals dangerously widen, and of necessity eventually project themselves into two ecological crises. The first, over-population,

is not the concern of this study; the second, non-target organisms being threatened by detrimental insecticide residues, is irrevocably juxtaposed with the purpose of this dissertation which involves itself in the research now in progress on new ways to eliminate detrimental insect populations without resorting to chemical agents which concomitantly have a deleterious effect on other populations--including man.

Research scientists now realize that procedures monitoring and pinpointing insect infestation sites are needed so that indiscriminate use of insecticides as preventive methods can be circumvented. This study purports to present the investigation of such a procedure: it looks toward an effective control of the sweet potato weevil Cylas formicarius elegantulus (Sm.) that does not resort to a dangerous use of chemical insecticides.

The sweet potato (Ipomoea batatas), a staple crop of Louisiana, is cultivated yearly on approximately 60,000 acres. The cash value of this crop is estimated to be twenty-two million dollars (\$22,000,000).<sup>1</sup> However, serious economic losses are sustained annually from the damage inflicted by

insects decimating the foliage and roots. Probably, the most destructive of these pests is the sweet potato weevil which feeds and oviposits on both the growing and stored crop.

The first official record of extensive crop destruction by the sweet potato weevil came from Neitner<sup>143</sup> who in 1857 reported that hundreds of acres of sweet potatoes were decimated in Ceylon. Approximately thirty years later, the presence of this insidious pest was noted in the New Orleans area.<sup>144</sup> The precise way in which the weevil was introduced into the United States is not known; however, it probably arrived here via the West Indies since both New Orleans and Ceylon ships docked there, receiving and delivering cargoes of sweet potatoes. At any rate, this insect is now located in a number of southern states: Texas, Florida, Mississippi, Alabama, and Louisiana.

Like many insects, the sweet potato weevil goes through the egg, larva, pupa, and adult stages. The eggs are deposited through the sweet potato skin and into the pith. They do no damage to the plant itself. Within four to fifty-six days, the larvae hatch by eating through the egg shells; subsequently, they tunnel within the pith itself. During their first

and second instar periods, the larvae bore towards the center of the pulp; in the third instar larval period, they sometimes burrow to the center itself. It is in this third period that the most extensive damage is done by the larvae to the sweet potato. In 12 to 154 days, the larvae pupate. The pupae, however, normally remain dormant for the 5 to 49 days required to form an adult. In an average of seven days, the adult emerges from the potato and has a life span of approximately 78 days (It is recorded that one male adult lived for 416 days).<sup>144</sup> Cockerham describes the adult in the following way:

The adult . . . of the sweet potato weevil is a snout beetle of antlike appearance. It has a narrow head and thorax, long legs, and a distended body. The elytra, or wing covers, are metallic dark blue, the head and snout dark blue, and the thorax and legs brick red. The long antennae are reddish brown, with a distal segment lighter and forming a thick club. In the male this club is slender, with parallel sides, and is longer than the total length of the remaining segments. In the female it is oval and shorter than the total length of the remaining segments. The snout is stout, slightly curved, and about twice as long as the head, with the antennae attached slightly beyond the middle. The antennae, tibiae, and tarsi are clothed with small bristly hairs. The claws are black. The adult is about one-fourth of an inch long.<sup>145</sup>

Shortly after emerging, adults mate and the female immediately begins to oviposit.

Extensive testing has been done to determine the host plants on which feeding and ovipositing take place.<sup>144</sup> In St. Landry Parish alone, over one hundred native plants were individually exposed to twenty weevils which either ate the plant or died. Over a number of years, it was determined that these insects feed and oviposit only on seventeen plants of the genus Ipomoea to which the sweet potato belongs; however, feeding alone was observed on other wild host plants. In the field, the weevil often gnaws the leaves and stem of the potato plant because of their accessibility. However, when portions of the root are obtainable, they puncture these by means of their long and slender proboscises. When possible, they avoid the light; thus feeding more often takes place on shaded surfaces, and whenever possible, on the root itself. For instance, five cages, each containing a healthy vine, one sweet potato, and four weevils, were checked at twenty-two day intervals. Seventy-five percent of the time, the weevils were on the potato; eleven percent of the time, they were on the vine.<sup>144</sup>

The sweet potato weevil has very few natural enemies. The major ones are parasites, nematodes, mites, Argentine ants, mice, birds, and two or three fungi. Unfortunately, none of these is effective in containing the weevil population.

The most effective control can be found in insecticides: one-half pound of Thiordan per acre is applied to the field; ten percent DDT at the rate of one and one-fifth ounces per bushel is dusted on the stored sweet potato. However, this insecticidal control, besides presenting potential ecological problems, has not been totally effective in eliminating the weevil. Therefore this research on the sex and food attractants of Cylas formicarius elegantulus (Sum.) has been initiated as an alternate and more felicitous method of effectively controlling the sweet potato weevil.

Such research requires the continuous availability of large quantities of these weevils. Since the sweet potato is a seasonal commodity and since the weevil requires this commodity in order to thrive, supply and demand are unfortunately seldom in accord.

Hammond,<sup>146</sup> in an attempt to rectify this situation,

has assiduously worked on the development of an artificial diet. To this point, he has not perfected one, and consequently, the circular problem associated with the supply of potatoes and weevils continues to manifest itself. In an attempt to partially ameliorate this situation, copious supplies of sweet potato peels were obtained from two seasonal canning companies with the hope that the peels themselves could be frozen and used for experimental purposes throughout the year. It was discovered (vide infra) that this was not a viable solution to the problem. Under these unavoidable, adverse conditions, research was nevertheless begun.

Hammond<sup>146</sup> had obtained data which indicate the presence of a pheromonal system in the sweet potato weevil. Twelve traps baited with virgin females were placed in the field for three days. At the end of this time, a total of 1,707 male weevils were caught.

It was shown in a number of instances<sup>142,143,146</sup> that the sex attractants of various insects were commonly present in their excrement. This seemed, then, a good place to begin the search for the pheromone of the sweet potato weevil, especially since previous work<sup>144</sup> on this insect had suggested that the males were excited when exposed to the



fecal matter of virgin females. For instance, in a one and one-half hour period, Hammond<sup>146</sup> was able to trap 820 male weevils in the field with excrement alone. A supply of the excrement from virgin females was provided<sup>147</sup> so that initial experimentation in an attempt to isolate the pheromone of the sweet potato weevil could commence. The extract of this frass decidedly elicited a response from the males; thus indicating that this first step was a move in a right direction. With the knowledge that in all but one of the recorded pheromonal isolations, only an infinitesimal amount of the attractant was present, scrupulous attention was given to the constant monitoring, not only of the extracted material itself, but also of the further manipulations associated with it. It was recognized that the compound or compounds which excited the male sweet potato weevils were potentially labile, and therefore, without assiduous surveillance, capable of being lost. Continuous bioassays provided this surveillance. For instance, concentrated extracts of the fecal material diluted with methylene chloride or hexane and subsequently placed on filter paper invariably aroused the male weevil. However, the extent to which the weevils

were excited apparently suggests that they are subject to a circadian rhythm: bioassays in the morning normally provoked a minimal response in that comparatively few insects reacted to the test material; assays from the identical batch of this material always optimally excited the same insects in the late afternoon. It was observed that this periodic behavior was not limited to the sex attractant alone. By day, there was a general lassitude; by night, a flurry of excitement. Corroborating evidence of interval activity also exists in the feeding response of these weevils. It was shown that their eating period is almost exclusively confined to the hours between twelve noon and twelve midnight.

A still unexplained phenomenon is found in the different responses of laboratory-reared and field insects to isolated sex attractants.<sup>40,146</sup> In certain instances, a supposed pheromone when tested in housed cages set the insects astir; when this same chemical was bioassayed outside, it enticed few insects. In these cases, the validity of the pheromone was questioned. In order to dispel any doubts about the actual presence of the weevil sex attractant in the extract from the frass, it was intermittently bioassayed

in the field where it allured a significant number of male insects.<sup>146</sup> With the knowledge, then, that the essence from the weevil excrement did indeed contain the sex attractant, chromatographic means were undertaken to purify it.

The first of these experiments was designed to isolate the pheromone by thin-layer chromatography, in which Silicar was the adsorbent. A bioassay of the intact chromatogram induced no activity. With the realization that Tumlinson<sup>85</sup> had experienced similar results because of the presence of multiple synergistic pheromones in the boll weevil, the plate was totally extracted and the extract, bioassayed. The lack of activity evinced in this bioassay indicated that Tumlinson's solution was not applicable here. It appeared, then, that perhaps the Silicar was responsible for a chemical modification<sup>148</sup> of the sex attractant; therefore, Alumina was substituted for Silicar as a separation adsorbent, with the thought that this might correct the anomaly. It did not. Subsequently, it was proved that if a drop of the active extract was placed on any adsorbent material, there was no biological response. This lack of response can perhaps be ascribed to the infinitesimal

amount of the pheromone actually present in the extract. When the extract is posited on an adsorbent material, it immediately disappears into the interstices. It would follow that if this disappearance were the reason for the loss of biological activity, re-extraction would recover the adsorbed pheromone; but it does not. This apparent failure, however, does not negate the feasibility of the proposition because the pheromone is present in miniscule amounts and re-extraction may be incapable of recovering a sufficient amount of active material to stimulate the insects.

Another possible reason for this loss of activity might be ascribed to the inherent fragility of the pheromone itself. For instance, Jacobson<sup>86</sup> claims that the pheromone of the American cockroach is unstable, even at cold temperatures, and decomposes in a few days if kept undiluted. It seems, however, that such a possibility can be discounted because the sex attractant of the sweet potato weevil maintains stability at temperatures as high as 170°C. After a residue containing the pheromone had been raised to this temperature in a vacuum, it was tested for biological activity by diluting it in hexane; a drop

of this dilution was placed on a piece of filter paper which was inserted into a feeding bin. The insects exhibited a flurry of excitement.

The possibility also exists that the disappearance of the pheromone might be attributed to air oxidation occasioned by the presence of a sensitive epoxide or a cis double bond<sup>149,150</sup>. It has been adequately documented (vide Section I) that a number of pheromones do possess cis double bonds and that one, the gypsy moth sex attractant, is an epoxide. Although the possibility of the phenomenon of air oxidation does in fact exist, and would be a plausible explanation for the loss of activity, the air oxidation should be more prone to occur if it were to be left in a basic solution in the open air. Therefore an experiment was designed such that an extract of the frass was treated first with an alkali solution in the air; then with an acid solution in the air. In both instances, activity remained. Risinger<sup>151</sup> confirmed that the weevil sex attractant is not easily air oxidized by allowing the attractant to remain untreated in the air for an extended period of time without a loss of biological attractancy.

When thin-layer chromatography failed, sublimation was turned to as a conceivable means of separation; and to some extent it worked. A residue from the fecal extract was sublimed at  $140^{\circ}\text{C}$  for three hours. The cold finger was removed and washed in hexane. The hexane solution was subsequently bioassayed by putting a drop of it on a piece of Whatman number one filter paper. Within seconds, the hexane had evaporated, and then the filter paper was manually waved inside the feeding bin. There was immediate agitation within the cage; the weevils en masse fluttered their wings and in general flew erratically. Some, however, actually lighted on the filter paper itself and attempted copulation. There was much more activity with the sublimate than with the crude fecal material, because one drop of the sublimed residue apparently contained a higher concentration of the pheromone than one drop of the unsublimed residue. It was supposed that mass spectrometry could offer a means of biogenetically classifying this material; then, through subsequent syntheses, the actual structure of the pheromone could be determined. The samples submitted for mass spectroscopy, however, were not sufficient to register a spectra. In order to obtain an adequate sample

of the extract for spectroscopy, mass rearing of the insect would be felicitous. This, however, was impractical, since no artificial diet exists.

In an effort to circumvent the impasse occasioned by a lack of weevils, a modified version of the milking process, designed by Yamamoto and used by Jacobson<sup>86</sup> on the American cockroach, was attempted. Approximately 500 virgin female weevils were placed in a Buchner flask. Air which had streamed over the weevils for 24 hours was directed through molecular sieve into a dry ice trap. By this process it was hoped that the pheromone would be wafted and finally congealed on the cold finger. During the course of this experiment, the cold finger continually had to be changed and extracted because ice repeatedly clogged it, despite the presence of potassium hydroxide before the air reached the weevils and molecular sieve after it had passed over them. The copious ice deposit was apparently not occasioned by the incapacity of the potassium hydroxide to dehumidify the air which entered the chamber; had this been so, the potassium hydroxide would have shown signs of excessive moisture. A more likely explanation is that the weevils themselves metabolically produced a large amount of water which quickly exhausted the molecular sieve.

Deposits on the ice finger were subsequently extracted three times with 100 milliliters of methylene chloride, which was then concentrated by flash evaporation to a volume of approximately one milliliter and tested for biological activity. If the pheromone were trapped, it did not register in the bioassay because of its miniscule presence. The molecule sieve was similarly extracted and bioassayed to ascertain if there was pheromonal activity associated with it. Again, there was none.

When this milking process did not yield a detectable pheromone, 127,000 whole body extracts of mixed sexes were tested for biological activity. There was no response. Three reasons for this lack of response suggest themselves: a masking compound was there, the sex attractant was lost in storage, or most probably, there were no virgin females present.

Both Jacobson et al.<sup>35</sup> and Roelofs et al.<sup>117</sup> demonstrated that a pheromone could be obscured by certain other chemicals which inhibit a biological response. In an attempt to determine if this phenomenon existed here, the crude material was subjected to Silicar column chromatography, eluting serially with petroleum ether, benzene, and ethyl acetate. More than 300 ten milliliter fractions were collected and



tested for biological activity. The assay was performed by depositing a few drops from each fraction onto a piece of filter paper and manually exposing the paper to the insects. None of the fractions produced activity. In the event that the ten milliliter solution had obscured the pheromone on the basis of high dilution, the solvent from each fraction was allowed to evaporate and the residue, in many cases hard to discern and possibly not even present, was bioassayed. The polyethylene beakers containing the residue were manually waved in the feeding bin. The insects did not react to them.

While this procedure did not definitively negate the possibility of masking, it suggested that the masking phenomenon was not working here. The second hypothesis, then, had to be considered; namely, that the sex attractant was lost in storage. Since the major source of live weevils was from sweet potatoes stored by farmers, and since infestations were periodic, supplies were uncertain. Therefore, when live weevils were available, normally in insignificant numbers (maybe millions are needed for meaningful results), the majority was placed in methylene chloride and stored for later mass extraction. Conceivably, a

chemical reaction occurred between the sex attractant and some other metabolic material. Such a process could deactivate the pheromone.

Since funding was totally inadequate, it was impossible either to hire needed personnel or to purchase vital equipment necessary to facilitate this research on the sex attractant. Nevertheless, the fundamental, yet indispensable, groundwork on the isolation of the weevil pheromone had been accomplished. With the pathway open to subsequent research on identifying the sex attractant,\* attention was turned to the weevil's food attractant in the hope that here, too, some fundamental progress could be made in the direction of isolating it.

Cockerham et al.<sup>144</sup> had already determined that the sweet potato weevil fed and reproduced on only seventeen plants of the genus Ipomoea. This selectivity indicated that some chemical or chemicals were

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\* Risinger and Hammond have subsequently narrowed the biologically active compound to a gas chromatographic peak which has been collected and awaits mass spectroscopy.

therefore uniquely present which attracted the weevils to these species. From the onset, it was recognized that isolation of the compound or compounds responsible for this selectivity would be difficult because of the presence of a number of biological subtleties, such as those categorized by Dethier:

. . . attractants (chemicals that cause insects to make oriented movement toward the source), arrestants (chemicals that cause insects to aggregate), stimulants (chemicals that elicit feeding phagostimulant, oviposition, etc.), repellents (chemicals that cause insects to make oriented movement away from the source), deterrents (chemicals that inhibit feeding or oviposition). Chemicals of plant origin may fit into any of these categories. Their action is most commonly associated with some link in the chain of behavior leading to ingestion or oviposition.<sup>152</sup>

Initial experiments were aimed at determining the specific part of the potato which attracted the weevils. After placing halved potatoes in a weevil infested bin, it was seen that the insects were not directed toward the exposed piths, but rather to the skins; thus a source material was localized which could be subjected to chemical analysis. Yet, in an effort to ascertain if the piths were in any way attractive, they alone were put into various containers with insects; in no case did feeding take place. Contrapuntally, only the skins were exposed to the

weevils to eliminate the possibility that there was a synergism between the union of pith and skin. The weevils totally embedded themselves within the skin; however, conceivably an exudate from the pulp was still present despite painstaking efforts to eliminate it.

Once it had been tentatively established that the attractant was contained in the skin, olfactometry<sup>153</sup> was used to put the bioassay on a quantitative basis. Fifty weevils of mixed sex were placed in an olfactometer and given the choice of a variety of combinations. For instance, when they were exposed to potato cube with skin and the extract of the skin, seven chose the cube; nine, the extract. Upon exposition to the pith and the skin, the reading was eight to nine; to the lyophilized skin and the natural skin, six to thirteen; to the lyophilized skin and the extract of the skin, thirteen to twenty; to the pith and lyophilized skin, three to eleven; to the pith and the pith plus water, two to twelve; to potato cube and water, twelve to fourteen; and so on. Perhaps the most meaningful data from these tests were found in the weevil's sensitivity to water. Ishikaya<sup>154</sup> in his study on Bombyx mori maxillary receptor cells demonstrated electrophysiologically that certain of

these cells are designed solely for the perception of water. Dethier<sup>152</sup> observed the same phenomenon in his work on the blowfly. Generally, however, the results of olfactometry were not sufficiently meaningful to determine if the attractant was present. Therefore, it was discarded as a viable means of monitoring an isolation scheme.

In a renewed effort to determine an effective method of bioassaying the food attractant, extracts from lyophilized skin of the sweet potato were applied to the pith with significant results. While it was not possible to count the bites because of the pulpous nature of the surface, a number of weevils had congregated on the pith and were apparently feeding. Upon examination, it was noted that the pith surface was similar to that of the skin when weevils were eating it, in that it was dehydrated and holes were visible. It was obvious that a delineative surface was essential to quantitate this feeding. A variety of such surfaces were tested; thus the extract was applied to the rind of vegetables (green beans, cucumbers, eggplants, etc.), which were subsequently placed in feeding bins. While the texture of these rinds was indeed suitable to register

puncture marks, none occurred. Perhaps, the presence of a phagodeterrent<sup>155</sup> in these particular vegetables might account for this lack of response. To circumvent the possibility that such a deterrent existed, filter paper became the medium on which the extract was placed. Like the vegetables, it too showed no response. However, when the filter paper was microscopically examined, it was noted that its fibrous texture did not, in reality, lend itself to the delineation of puncture marks. Corks, on the other hand, did.

On two number ten corks were painted approximately ten milligrams of the residue from lyophilized skin extract. These, together with the insects, were placed into cardboard containers of varying sizes. When it was observed that the insects did not respond under these conditions, the corks were then put into the feeding bins themselves with the hope that the weevils might respond in their pseudo-natural environment. They did. It was now possible to monitor the feeding response quantitatively, keeping in mind the fact that the behavior of the insects in their pseudo-natural environment had to be weighed. Therefore, specific amounts of extract were applied

to specific sizes of corks which were positioned in a specific environment, namely, the insect feeding bins. For instance, a minimum of 500 number ten corks were painted with 2.4 milligrams of concentrated filtrate and put into a feeding bin. When they were examined microscopically, they invariably registered an average biting response of 400. The initial amount (2.4 milligrams) of the concentrated filtrate was then diluted tenfold, and subsequently, a hundredfold. When these solutions were painted on number ten corks and placed into a feeding bin, the biting response ranged from 50 to 100 on the tenfold dilution, and from 1 to 5 on the hundredfold. Then, 2.4 milligrams of the concentrated filtrate were painted onto five corks of varying sizes, i.e., numbers 2, 3, 4, 5, 6, and 8. After placing these corks into feeding bins, they were checked for biological response. The average biting response of cork numbers two through six was approximately 150; on cork number eight, the average response level had risen to approximately 250; and as was previously noted, the average biting response on cork size ten was 400. It appears that as the size of the cork or the amount of the extract decreases, so also does the average biting response decrease.

This seems to indicate that a complexity exists in the insect-host plant interrelationship; thus it was imperative to adhere strictly to a specific bioassay in order to obtain consistent and meaningful results.

In order to determine the degree of importance of the weevil's tactile probing of the cork's surface, a film of paraffin was applied to the surfaces of number ten corks to separate the surfaces themselves from the tactile receptors of the insects.<sup>156</sup> These waxed corks were painted with 2.4 milligrams of the extract from lyophilized skin, and placed into a feeding bin. Subsequently, they were examined microscopically, and it was noted that the average biting response had been lowered to 50. This again suggested that the feeding response is subject to modulation, not only by semiochemicals,<sup>157</sup> but also by tactile reaction to the surface of the host plant.

A resistant strain<sup>158</sup> of the host plant (W-15-2)<sup>159</sup> was then acquired in an effort to gauge the weevil's reaction to its surface which had been treated with the extract from lyophilized skin. Half of this surface did not contain the extract; the other half was painted with 4.8 milligrams of it. After placing this potato in the bin, it was noted that the



weevils infested the test side, while at the same time, they showed no interest in the untreated skin. When the potato was microscopically examined, there was evidence that the weevils were feeding. Again, because of the texture of the skin, it was impossible to quantitate these bites. The control side showed no evidence of feeding; however, the feeding response, unless manifold, was difficult to discern. Next, 4.8 milligrams of the extract were painted in the form of a cross on the surface of this resistant strain. When the weevils were exposed to the potato, they aligned themselves on the cruciform. Microscopic examination showed heavy feeding along the lines of the cross; while the rest of the potato did not exhibit noticeable feeding. These experiments suggest that the extract contains a luring chemical, absent from the resistant strain; or, if this chemical is present in the resistant strain, it is there in low concentration. Possibly its presence could be negated by a phagdeterrent.<sup>155</sup>

In an effort to circumvent problems that might arise from the absence of fresh sweet potatoes, industrial sources were turned to with the hope that the peels which they could furnish, would offer a large and continuous source to be extracted. Now that

a bioassay had been successfully found, these large supplies of peels, easily frozen, could be readily tested to determine if they were a viable source. The Bruce Company furnished a sample batch of alkali treated peels which had a pH of 12. A one milliliter aliquot of this material when applied to corks, elicited no response; therefore, one liter was brought to a pH of 6 and extracted with methylene chloride. The remaining water layer was bioassayed and proved negative. Subsequently, the methylene chloride was concentrated, and it too was tested for biological activity. There was none. Next, the King Phaer Company supplied steam treated peels. Some of these were lyophilized and extracted. Four milligrams of the extract were applied to five corks and bioassayed. There was an average of six bites per cork. When 2.4 milligrams of the extract from fresh peels were applied to corks, the average biting response was 400. Perhaps, the food attractant in the King Phaer supply was steamed distilled at the plant. It is also possible that the high temperatures employed in steaming the peels away caused the majority of the food attractant to become inactive. At any rate, it

was felt that neither the alkali nor steam treated peels could offer a solution to a diminishing supply of fresh potatoes.

Thick layer chromatography was innovated to ascertain if the insects could be induced to feed on a specific band. There was precedent for this move in the studies Sakan made on the lacewings.<sup>109</sup> After having applied approximately 200 milligrams of the residue from lyophilized skin extract, the plate was eluted with a petroleum ether, ethyl acetate, benzene (90:5:5) solvent system. Five bands were seen upon examination of the plate by means of ultraviolet light. The entire plate was then positioned into a feeding bin and periodically checked to determine if any insects had amassed along a particular zone. There was no evidence of this. Subsequently, the plates were withdrawn to see if any puncture marks were present. There were none. The lack of feeding response could perhaps have been caused by the texture of the surface on the plate, or it might suggest the presence of a multiple, synergistic food attractant system which had been separated in chromatography and consequently deactivated. To test these two hypotheses, the individual bands were removed and

extracted. After concentrating the extracts, they were individually applied to number ten corks. Four of the bands elicited no biological response; however, the cork which had been painted with the extract from band two (Rf 0.07) had approximately 180 bites. These results again reinforce the notion that the texture of the surface is biologically important; and while they do not completely dismiss the possibility of multiple synergism, they do indicate that if such complexity were present, the compounds involved were at least similar. In contrast, the food attractant system of the Bombyx mori<sup>100</sup> is composed of a synergistic system of divergent chemicals which would not have elicited a positive response if they had been subjected to chromatography. This isolation method would have been continued; however, it was impractical to do so because of the large amount of material that had to be separated. Column chromatography, on the other hand, was a technique more suitable for the task of separating mass amounts of crude material.

The crude material was therefore subjected to Silicar column chromatography. The eluting solvent was serially increased in polarity from petroleum ether to methanol. Twenty milliliter fractions were taken

over a five-day period. Each fraction was concentrated and applied to the surface of a cork which was positioned in a feeding bin. None of the fractions showed any feeding response. Additionally, all of the individual fractions were recombined and tested for biological activity. Again, the corks showed no puncture marks. Conceivably, the necessity of allowing the food attractant to remain on the column for an extended period of time, deactivated it.

It now seemed important to test the chemical stability of the food attractant in the concentrated extract in order to determine if a classical isolation scheme employing acid and base could be used. Approximately 20 milligrams of concentrated extract were put into 6N, 3N, and 1N hydrochloric acid, respectively, for three hours. In each instance, half of the volume was then brought to a pH-7; the other half, left acidic. Both the neutral and acidic fractions were painted on corks and bioassayed. No biting responses were recorded. In order to test the stability of the compound in alkaline conditions, 20 milligrams of the extract was treated with 6N, 2N and 1N sodium hydroxide for three hours. Again, half of the volume was brought to a pH-7; the other half, left alkaline. Bioassays of both fractions indicated the

absence of a feeding response. In order to obviate the possibility of air oxidation, the extracts were put into an alkaline solution under a nitrogen atmosphere. Here again bioassay of the neutralized material was negative. Apparently, then, the food attractant, unlike the sex attractant, lost its biological activity when exposed to base or acid treatment. This remission can possibly be attributed to the presence of a chemical possessing labile functional groups, such as cis double bonds or sensitive esters.<sup>150</sup> At any rate, an isolation scheme involving the use of acid or base was therefore ruled out.

Since the method of sublimation developed in this study successfully separated the active material associated with the sex attractant from a large quantity of inert material, it was felt that this same method might be advantageously applied in an attempt to isolate the food attractant. If it worked, the active compound, although still impure, could be disjoined from an extraneous mass of crude material. Therefore, 500 milligrams of the residue were sublimed. When 1.3 milligrams of the sublimate were removed from the cold finger and tested for biological activity, 258 bites were counted.

By this time, a Perkin-Elmer 990 Gas Chromatograph coupled to a Hitachi Perkin-Elmer RMS-4 Mass Spectrometer was available, thus presenting a method of separating and analyzing the sublimate itself. On the first occasion in which the sublimate was subjected to gas chromatography/mass spectrometry, only two prominent peaks were seen on the chromatograph; the remaining peaks were insignificant in size. The mass spectrometer furnished data on these two prominent peaks alone. The first compound from one of these showed an  $m/e$  of 220; the base peak corresponded to 205 mass units. These data indicate the loss of a methyl group. That there were few peaks corresponding to multiple cracking patterns indicated that the molecule was probably annuloid, and more than likely, aromatic. Correlation of the relative intensities of the mass units present from the first spectrum with those from 2,6-di-*t*-butyl-4-methylphenol suggested that they were the same. This phenolic compound is a widely used anti-oxidant. The second prominent compound seen on the gas chromatograph yielded a mass spectrum which had a parent and base peak corresponding to an  $m/e$  of 94. Here again there was little cracking, suggesting that the molecule was annular and aromatic in structure.

It was ruled out that the compound was an alkyl substituted aromatic because of a lack of the tropilium ion at 91 mass units. The splitting out of a unit with an  $m/e$  of 28 indicated the loss of carbon monoxide. All of these data suggested that the parent compound is phenol. Comparisons of peak intensities with a standard spectrum corroborated this.

It was difficult to justify the anti-oxidant as part of a biological system; there seemed to be no biogenetic precedent for it. A reasonable explanation for its presence could be found in the fact that the sublimate dissolved in methylene chloride had been placed in a polyethylene beaker; the methylene chloride could have extracted this compound from the polymer. When the process was repeated without resorting to a polyethylene beaker, the stabilizer did not manifest itself. This experience again re-emphasized the caution that must be exercised in dealing with miniscule amounts of a food attractant.

Phenol, the other compound identified, was bioassayed and evoked a positive response. Although there was multiple biting, there seemed to be a difference in the way the insects fed on the corks. The puncture marks, unlike those provoked by the normal



extract, were comparatively larger and deeper. This experiment was repeated a number of times with similar results. It is difficult to adjudge with any certitude the reason for this different feeding behavior.

Additionally, the feeding response could be attenuated by lowering or raising the concentration of purified phenol. When five milligrams of purified phenol<sup>160</sup> were painted onto number ten corks which were then exposed to the weevils, the biting frequency averaged 300. On the other hand, bioassays at the level of 0.5 milligrams per cork elicited an average biting frequency of 22; at 0.05, the average frequency was one. When the level was raised to 50 milligrams per cork, again the biting frequency was diminished to an average of three. The response upon lowering the concentration seemed reasonable since the same phenomenon existed when the normal extract was diluted. Similarly, the attenuation at higher concentrations brings to mind the research of Dethier on the Fly.<sup>97</sup> As he raised the concentration level, the food attractant concomitantly became a repellent. With Dithier's research in mind, approximately 50 milligrams of phenol were placed on a cotton swab and manually moved into the vicinity of the weevils. Their motion

was immediately arrested, and their antennae showed signs of exacerbation. Their forelegs engaged in a wiping motion on their antennae.

Upon repeating the gas chromatography/mass spectrometry, it was discovered that phenol was apparently not intrinsically associated with the sublimate because its presence was not observed. Conceivably, it was accidentally acquired as an impurity, since in the first sublimate, it was one of the two most prominent peaks. Nevertheless, this probable accident resulted in a fortunate acquisition, because regardless of its source, phenol did elicit a positive biological response. Immediately, other phenolic type compounds were bioassayed: p-cresol, o-cresol, p-nitrophenol, catechol, resorcinol, phloroglucinol, pyrogallol, gallic acid, tannic acid, coumarin, 3,4-dihydroxybenzaldehyde, methyl salicylate, salicylic acid, 4-t-butylcatechol, 5-t-butyl-1,3-benzodioxole, 3,4-diacetoxybenzaldehyde, 4-hydroxymethylcatechol, anisole, 4-hydroxymethylcatechol 1,2-diacetate, 4-bromo-6-t-butyl-1,3-benzodioxole, 4-vinyl-6-t-butyl-1,3-benzodioxole, and salicylaldehyde. Five milligrams of each of these compounds were painted on three corks. There was not only a lack of response, but even

a suggestion of repellence. Under normal circumstances, corks painted with inactive compounds were spotted with fecal pellets; however, those painted with some of these phenolic type compounds, such as p-cresol, o-cresol, anisole, and resorcinol, showed no such signs.

Through subsequent mass spectral analysis of the sublimate, nine aliphatic methyl esters (methyl tetradecanoate, methyl pentadecanoate, methyl hexadecanoate, methyl heptadecanoate, methyl octadecanoate, methyl octadecdienoate, methyl octadectrienoate, methyl docosanoate, and methyl eicosanoate), two straight chained hydrocarbons (n-heneicosane and n-tricosane), and squalene were identified and confirmed. Each was tested in the following manner: Four milligrams of the respective compound were painted onto three corks which were exposed to the weevils. When the corks were observed microscopically, there was no evidence of feeding. The same procedure was used to test these compounds jointly. A mixture composed of one milligram of each of the compounds was applied to corks which were exposed to the weevils. Again, there was no evidence of feeding. Since these methyl esters may

have been formed as a result of esterification while the skins were being extracted with methanol and methylene chloride, they were hydrolyzed and the acids were bioassayed. Again there was no activity. The possibility still existed that these esters were in some way associated with the food attractant. Since they were conceivably formed via transesterification, the lyophilized skin was extracted first by using only methylene chloride; then by substituting ethanol for methanol.

In the first case, 2.5 milligrams of the methylene chloride extract were painted onto corks which were exposed to the weevils. When examined microscopically, the corks showed an average biting response of 240. In the second case, 2.5 milligrams of the methylene chloride and ethanol were applied to corks and bioassayed. The average feeding response was 215. However, mass spectral data of the sublimate did not indicate the presence of methyl esters. Nonetheless, it must be noted that no compounds were identified from these data because of hydrocarbon fragmentation which did not allow assignment of a parent peak. This procedure still did not definitively prove the absence of the methyl esters from the skin because they may be

intimately associated with cellular structure.<sup>163</sup>

Perhaps methylene chloride or methylene chloride and ethanol were not capable of releasing them.

With the knowledge that ethanol extraction of plant material was proffered as the reason for the appearance of ethyl glycosides,<sup>161</sup> that the phenomenon of transesterification could be responsible for the appearance of the respective methyl esters, and that phenol was the only compound which showed biological activity, it was decided to explore the possibility that phenolic esters might evoke a biological response. It was therefore necessary to synthesize a series of phenolic esters. After the C<sub>2</sub>-C<sub>10</sub>, C<sub>12</sub>, C<sub>14</sub>, C<sub>16</sub>, and C<sub>18</sub> phenolic esters had been synthesized, five milligrams of each were applied to corks and bioassayed. The results showed that C<sub>2</sub> had an average biting response of 59; C<sub>3</sub>, 41; C<sub>4</sub>, 22; C<sub>5</sub>, 1; C<sub>6</sub>, 0; C<sub>7</sub>, 6; C<sub>8</sub>, 15; C<sub>9</sub>, 3; C<sub>10</sub>, 0; C<sub>12</sub>, 0; C<sub>14</sub>, 0; C<sub>16</sub>, 0; and C<sub>18</sub>, 0. When the chain length was longer than C<sub>9</sub>, no activity could be evoked. There was a small amount of activity associated with the C<sub>7</sub>-C<sub>9</sub> phenolic esters; and a positive gradient level of biological response from the C<sub>4</sub> phenolic ester to phenol itself. It is improbable that the response in the short chain phenolic esters

results from the presence of a phenol impurity, because the esters from  $C_2$  to  $C_{12}$  were distilled twice, and subsequently subjected to Silicar chromatography before they were applied to the corks. Even if there were a small amount of phenol impurity from hydrolysis caused by the water in the air, the impurity would have had to have been less than 0.05 milligrams of the applied sample. If it were greater than this, it would have shown up as an impurity in the NMR. Gas chromatography would also have easily registered an impurity of one percent. It did not. Additionally, an attenuation experiment of phenol (vide supra) showed that the feeding response had almost completely abated at 0.05 milligrams. More likely, the biological response evoked by these esters was occasioned by their ability to still fit into an enzymatic site which is responsible for the triggering of a neurorelay system<sup>57</sup> within the insect. The  $C_6$  and  $C_{10}$ - $C_{18}$  phenolic esters conceivably do not fit into this enzymatic site and, consequently, cannot elicit a biological response. Another possibility for this variant biological response is that an enzyme system of the antenna might be hydrolyzing the short chained phenolic esters, thereby releasing phenol. Such a

system would probably have an active site which is hydrophilic in nature, and consequently, more prone to hydrolyze the short chained esters and repel the hydrophobic long chained esters.<sup>162</sup>

The same fundamental problems which beleaguered research on the sex attractant were present throughout this attempt to isolate the food attractant. There was inadequate manpower to raise and sustain continually an insect population; a lack of funding obviated the early purchase of requisite equipment; and sweet potatoes were not sufficiently available. It is true that large quantities of these potatoes were seasonally acquired, but there was no place free of weevils in which to store them--nor was there sufficient help to skin them before they were ruined either by frost, black rot, fungus, rodents, or the insects themselves. In an attempt to circumvent the problems associated with storage and skinning, supplies of alkali and steam treated peels were obtained from industrial sources. Unfortunately, these sources did not offer a viable product, and the problem still remained.

Nevertheless, a considerable amount of essential work has been done in the process of isolating both the sex and food attractants of the sweet potato weevil.

Crude material containing the pheromone has now been refined to a stage where it awaits gas chromatography/mass spectral analysis. It is possible that this technique will culminate in a final isolation and structure elucidation of the sex attractant. With synthesis, this compound could be spread through sweet potato fields where it could interfere with the sexual communication system of the weevil. It could also be employed as a monitoring device to determine infestation sites, and thereby govern the amount of insecticide to be applied. The possibility exists that a synthetic pheromone could be incorporated into a wafer treated chemically<sup>142</sup> to destroy the insects which would be drawn to it.

Likewise, the isolation and structure determination of the food attractant are closer to being realized now that this study has been completed, essentially because an effective method of bioassaying the presence of the food attractant has been found. Such a method has demonstrated that phenol and to a lesser extent some short chained phenolic esters do induce the insects to feed. When the compounds responsible for feeding and ovipositing are eventually isolated and identified, a phylogenetic program<sup>159</sup> will be given a



forward thrust. It could rapidly be determined if incipient plants possess these stimulants. Those that did, could be eliminated from a genetic pool. It would also be feasible to incorporate a synthetic food attractant into nutritive artificial diets.

In general, this research on the sex and food attractants of the sweet potato weevil has offered the initial and vital steps toward an alternate and more felicitous method of an effective control that might eventually lead to a reduction in the use of insecticides.

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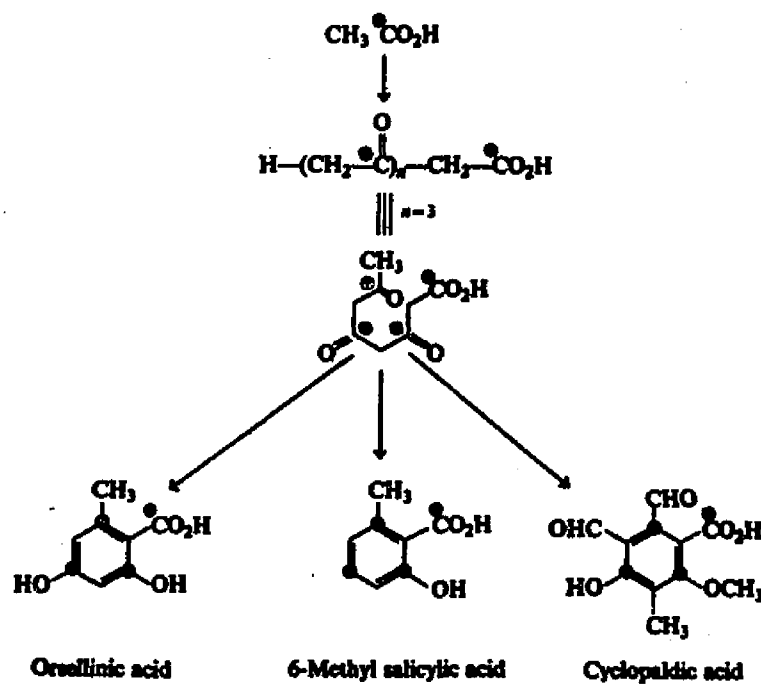
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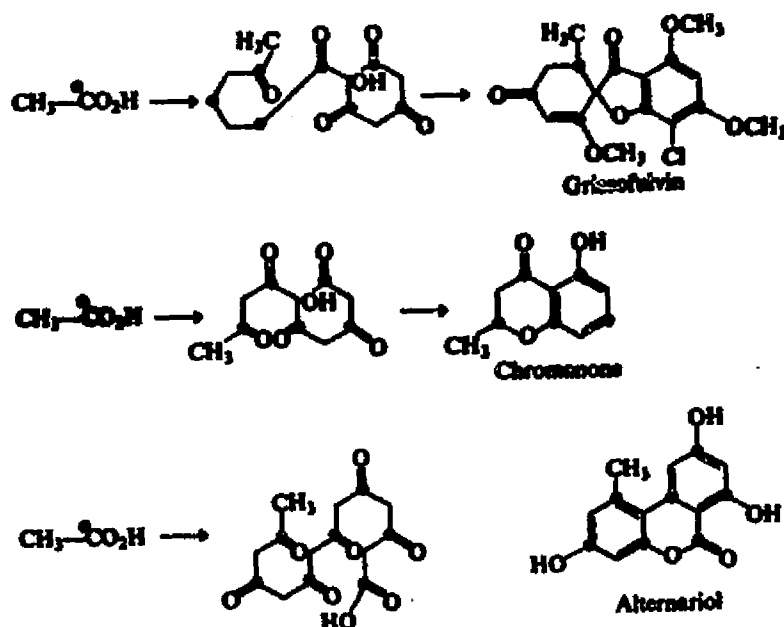
## APPENDIX

ATTEMPTED SYNTHESIS OF PREPHENIC ACID AND STUDIES  
DIRECTED TOWARDS FUNCTIONALIZATION AND UTILIZATION  
OF ENDO-TRICYCLO [6.2.1.0<sup>2,7</sup>] UNDEC-9-EN-3,6-DIONE

In nature there are two basic pathways which must be travelled in order to arrive at aromatic compounds. One of these involves the "acetate hypothesis."<sup>1,2</sup> According to this hypothesis, acetate sequentially condenses to form the poly- $\beta$ -keto intermediate, which subsequently cyclizes to produce aromatic rings having meta-oriented hydroxyl groups.

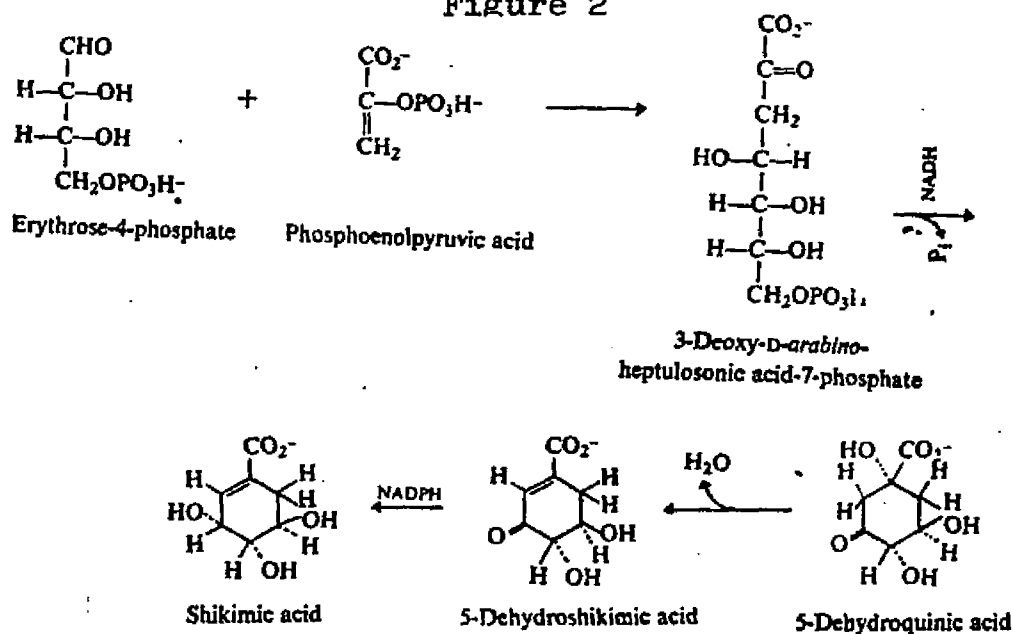
Figure 1

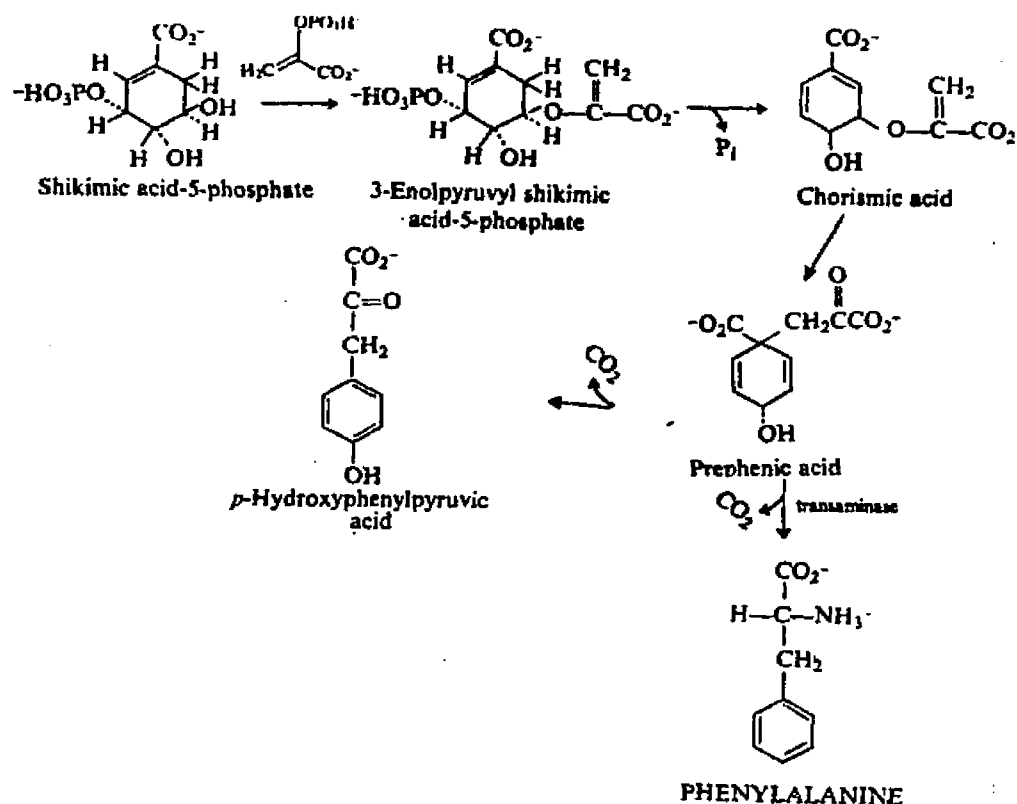




The second is the shikimic acid pathway.<sup>3,4</sup> Here, most of the other aromatic compounds and a number of alkaloid classes find their origin.

Figure 2





The immediate precursor of the aromatics is prephenic acid, which was first isolated<sup>5,6</sup> as its barium salt from mutant strains of Eschericia coli.

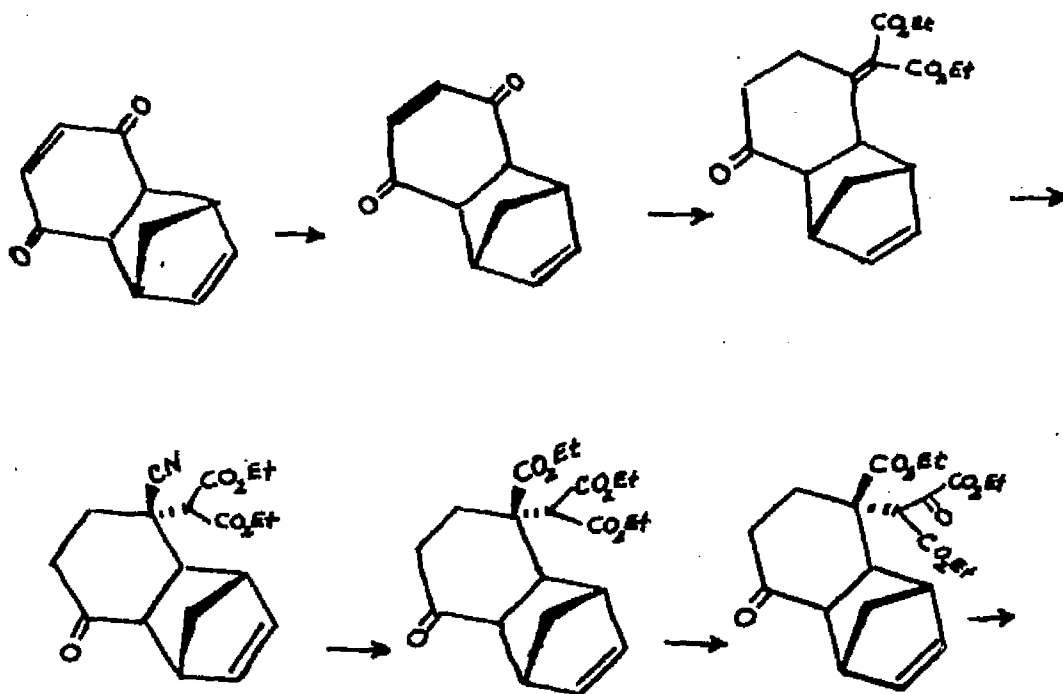
Prephenic acid, a fragile bio-molecule, has not yet been synthesized in yields sufficient to be labelled and subsequently used in the probe of its biogenetic descendants. Plieninger's synthesis,<sup>7,8</sup> although stylistically designed, contains one major weakness: it introduces two double bonds after the basic nucleus has been constructed. The method by which he introduces these two double bonds forces him into an untenable position. When the inherently labile molecule is

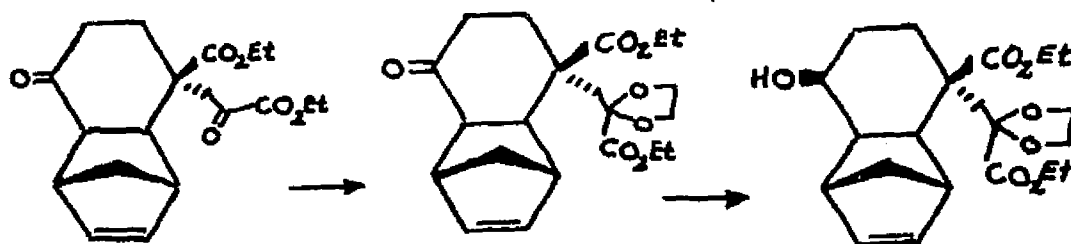


exposed to either acid or base conditions, it decarboxylates because of steric strain associated with the tertiary carboxyl group and because of the tendency of the cyclohexadienol nucleus to aromatize.

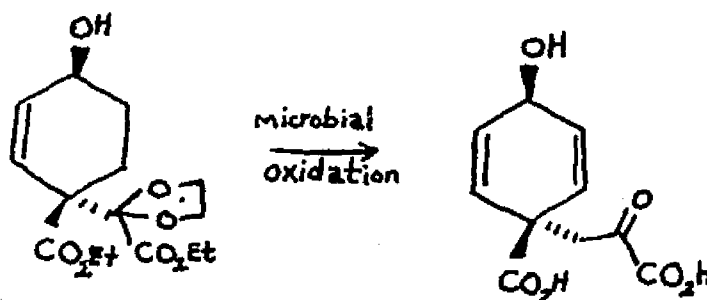
This problem of aromatization might be partially circumvented by resorting to a starting material which possesses latent functionality, e.g., a hidden double bond. Such a starting material can be found in the reduced Diels-Alder adduct, endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3,6-dione, referred to in this study as "the dione." The synthesis envisioned is as follows:

Figure 3





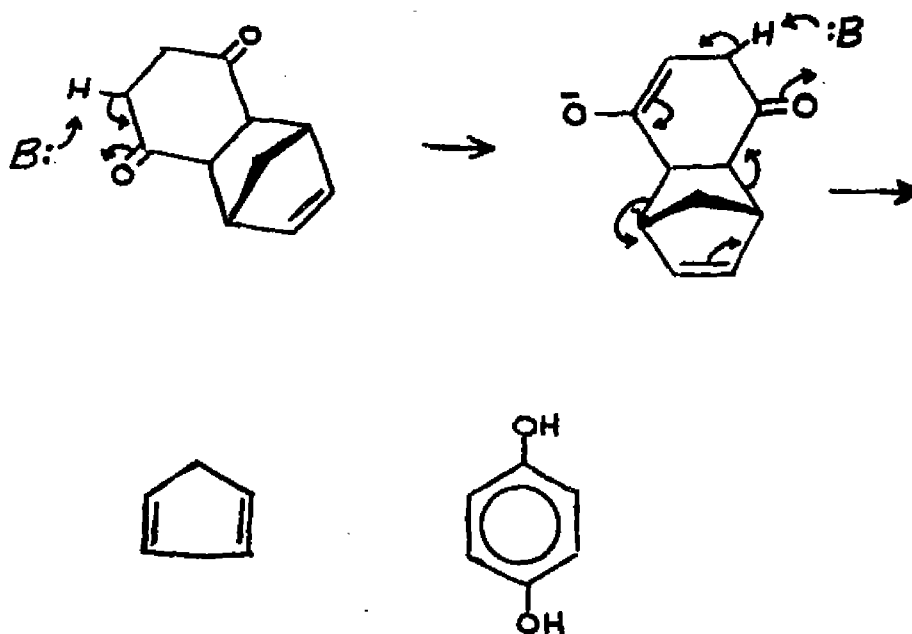
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It was felt that the formation of the  $\alpha,\beta$ -unsaturated cyano-acetic ester, the first step in the synthesis to be tested, could be accomplished by a Cope<sup>10</sup> modification of a Knoevenagel<sup>11</sup> reaction. That the dione itself showed no tendency to exist as its aromatic tautomer strengthened the feeling that the double bond, once formed, would not migrate into the six membered ring. Logically, the Knoevenagel reaction should have worked; unfortunately, it did not. Therefore a base catalyzed condensation was considered.<sup>12</sup> In this instance, the dione ether did not react or underwent a retro-Diels-Alder reaction.

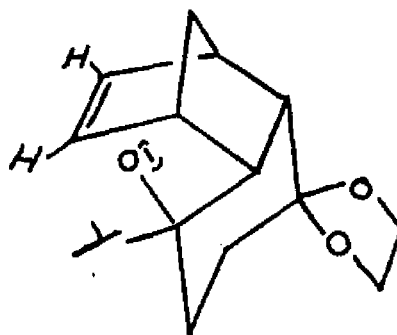
The mechanism for this retro-Diels-Alder reaction could be ascribed to the following:

Figure 4



A possible means of preventing this retro-reaction lay in blocking one of the ketones as its ethylene glycol ketal. The block was effected, and again, Knoevenagel and base catalyzed reactions were tried. No condensations occurred; or, if they did occur, they too retrograded, because of the steric strain associated with the transient formation of the endo-hydroxyl group (Figure 5).

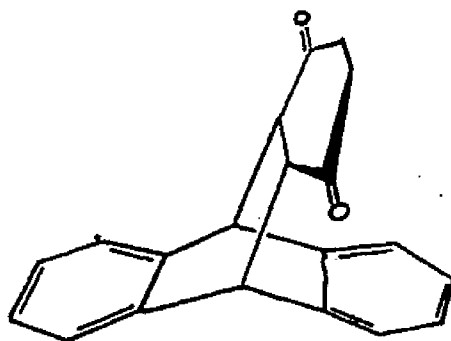
Figure 5



It was now assumed that this retro-condensation could be circumvented by resorting to a Reformansky reaction;<sup>13</sup> this too failed. It was decided that the ketal might be responsible for the failure of these condensation reactions. The free ketone was therefore reduced to a hydroxyl group. When the latent carbonyl group was unveiled, it produced endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3-ol-6-one. Unfortunately, condensations on this compound also failed.

The chemistry of these systems obviously presents problems which conceivably arise from the norbonyl group. With this thought in mind, the norbonyl group was replaced by the anthracene moiety (Figure 6).

Figure 6



When a condensation was then attempted, a retro-Diels-Alder reaction occurred. This new sequence seemed to compound the problems and the synthesis of prephenic acid was abandoned.

Work on the dione system, however, suggested that if the dione could be annulated, a synthesis

of phyllocladene might subsequently be effected. Earlier attempts by Green<sup>14</sup> and Aaron<sup>15</sup> to annulate the dione had failed. Now there was an attempt to determine if the C<sub>4</sub> position of the dione could be alkylated or activated. If it could be activated, then alkylation and annulation would possibly ensue. Attempts at carboxylation or formylating failed. Finally, the elusive annulation of the dione was accomplished by resorting to an acid catalyzed annulation.<sup>16</sup> However, the product from the annulation of the dione was obtained in small yield. There is still need to find the best conditions to raise the yield, so that the synthetic sequence can be continued.

Many attempts have been made to functionalize or selectively convert the dione and its descendants into utilizable intermediates. These attempts are indicated in Figure 7 and subsequently described in detail.



Preparation of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undeca-4,9-dien-3,6-dione<sup>17</sup>

While keeping the distillation temperature between 41-45°C, cyclopentadiene was collected in an ice cooled flask.<sup>18,19</sup> Thirty-three grams (0.5M) of freshly distilled cyclopentadiene were added dropwise into a one liter Erlenmeyer flask containing a magnetic stirring bar, 500 milliliters of 95% ethanol, and 54 grams (0.5M) of p-benzoquinone. After the addition of cyclopentadiene was completed, the dark solution was allowed to stir for another 12 hours. At this time, the solvent was flash evaporated until the saturation point was reached; whereupon, the solution was cooled and crystallization ensued. The crystals were collected and redissolved in diethyl ether and Norit-A was added. After bringing the solution to reflux, it was filtered through a pad of Celite and Alumina. The ether was then flash evaporated, and yellow crystals were collected. The spectra showed the compound was identical to endo-tricyclo[6.2.1.0<sup>2,7</sup>]undeca-4,9-dien-3,6-dione. The presence of the triplet at 6.1 ppm corresponding to 2 protons and the singlet at 6.5 ppm corresponding to 2 protons was corroborative.



Preparation of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3,6-dione<sup>20</sup>

Into a 500 milliliter Erlenmeyer flask containing a magnetic stirring bar, two grams (0.05M) of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undeca-4,9-dien-3,6-dione, and 100 milliliters of water were added five milliliters of glacial acetic acid, and subsequently, 15 grams (0.25M) of zinc dust. This mixture stirred for two hours; whereupon, the zinc was removed by filtration, then washed with diethyl ether. The filtrate was saturated with sodium chloride and extracted three times with ether. The combined ether extracts and washings were flash evaporated and an oily residue remained. Chloroform was added to the residue and if any precipitate formed, it was filtered. The chloroform was then flash evaporated and a pale yellow oil remained. This oil was identical to a sample prepared by C. Aaron.<sup>21</sup> The yellow oil acquired weighed 5.4 grams corresponding to 68 percent yield. Infrared absorbance at 5.8 microns confirmed the presence of the ketones. The occurrence of a symmetrical 16 line pattern NMR from 2.0-3.0 ppm and the loss of the singlet at 6.5 ppm assured the product.

Preparation of the ethylene glycol mono-ketal  
of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3,  
6-dione

Into a 300 milliliter round bottom flask fitted with a West condenser on a Dean-Stark apparatus were poured 100 milliliters of benzene. To this solution were added 51.3 grams (0.29M) of the dione, 18.0 grams (0.29M) of ethylene glycol, and 0.5 grams of p-toluenesulfonic acid.<sup>21</sup> The reaction mixture was refluxed until all of the water had azeotroped. The benzene solution was then cooled and flash evaporated. A resulting oily residue was subsequently dissolved in diethyl ether and washed with Na<sub>2</sub>CO<sub>3</sub>. The ether was then dried over CaCl<sub>2</sub> and flash evaporated. The oily residue was triturated with petroleum ether which induced crystallization. The crystals melt at 59-60°C. The mono-ketal is acquired in quantitative yield. Elemental analysis of the mono-ketal: theoretical C, 70.90; H, 7.29; found, C, 70.64; H, 7.34. A strong infrared absorbance at 8.9 microns coupled with NMR showing a singlet corresponding to 4 protons at 4 ppm and the loss of the symmetric pattern at 2-3 ppm indicated the formation of the mono-ketal.

Preparation of the ethylene glycol mono-ketal of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3-ol-6-one

Into a 125 milliliter Erlenmeyer flask containing a magnetic stirring bar and 40 milliliters of ethanol, were added 2.2 grams (0.01M) of the ethylene glycol mono-ketal of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3,6-dione. Into this mixture were inserted 0.37 grams (0.01M) of sodium borohydride.<sup>22</sup> The mixture was allowed to stir for 12 hours; whereupon, ten milliliters of water were added. The volume of the solvent was then reduced by boiling to approximately 15 milliliters. After cooling, the solution was extracted with diethyl ether. The diethyl ether was dried over MgSO<sub>4</sub> and flash evaporated. The remaining viscous oil was subjected to spectral analysis which showed that the presence of the carbonyl group had disappeared. The viscous oil weighed 1.98 grams which corresponded to a 90 percent yield. The appearance of a strong O-H stretch at 2.9u and loss of absorbance at 5.8u coupled with 4 proton absorbance 4 ppm was corroborative.

Hydrolysis of the ethylene glycol mono-ketal of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3-ol-6-one

Into a 125 milliliter Erlenmeyer flask containing a magnetic stirring bar and 40 milliliters of a 5% solution of oxalic acid were added 2.2 grams (0.01M) of the ethylene glycol mono-ketal of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3-ol-6-one. The solution stirred for forty-five minutes. It was then washed with Na<sub>2</sub>CO<sub>3</sub> and extracted three times with diethyl ether. The diethyl ether solution was dried over MgSO<sub>4</sub> and flash evaporated. Spectral analysis confirmed the presence of a carbonyl group which had not been seen in the parent compound. That the hydrolyzed compound was in fact endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3-ol-6-one was established when it was transformed via bromination into its bromo-ether, a compound synthesized by Green.<sup>22</sup> The endo-alcohol acquired weighed 1.6 grams, corresponding to a yield of 95 percent. The reappearance of the carbonyl group at 5.8u, and the loss of the absorbance at 4 ppm suggested hydrolysis.

Attempted preparation of the ethylene dithiol mono-ketal of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-en-3, 6-dione

Into a 300 milliliter round bottom flask fitted with a West condenser on a Dean-Stark apparatus were poured 100 milliliters of benzene. To this were added ten grams (0.06M) of the dione, six grams (0.06M) of ethylene dithiol, and 0.5 grams of p-toluenesulfonic acid. The reaction mixture was refluxed for 12 hours. The benzene solution was then cooled and extracted three times with Na<sub>2</sub>CO<sub>3</sub> solution. The organic layer was dried over CaCl<sub>2</sub> and flash evaporated. The oily residue was shown to be a mixture of the starting materials.

Attempt 2. All conditions were the same as in attempt 1, except that H<sub>2</sub>SO<sub>4</sub> was used as a catalyst. The reaction failed.

Attempt 3. All conditions were the same as in attempt 1, except that five equivalents of ethylene dithiol were employed. This attempt was unsuccessful.

Attempt 4. All conditions were the same as in attempt 3, except that toluene was substituted for benzene. This also failed.

Attempt 5. All conditions were the same as in attempt 1, except that borontrifluoride<sup>23</sup> was employed as a catalyst. This attempt also failed.

Attempted synthesis of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3-acetocarboxethylmethylen-6-one

Attempt 1. Into a 100 milliliter round bottom flask equipped with a West condenser fitted with a Dean-Stark apparatus and containing 25 milliliters of ethanol were added 5 grams (0.026M) of dione, 3.38 grams (0.026M) of ethyl acetoacetate, and 20 drops of piperidine.<sup>24</sup> The reaction was run for 15 hours at 0°C. At the end of this time, the solution was poured into 100 milliliters of diethyl ether. This organic layer was washed with ammonium chloride solution and dried over Na<sub>2</sub>SO<sub>4</sub>. It was then flash evaporated. Spectral analysis showed that the reaction had failed.

Attempt 2. All conditions were the same as in attempt 1, except that ammonium acetate was substituted for piperidine. This attempt also failed.

Acid catalyzed annulation<sup>25</sup> of endo-tricyclo  
[6.2.1.0<sup>2,7</sup>]undec-9-en-3,6-dione

Into a 200 milliliter round bottom flask equipped with a condenser were placed 20 grams (0.11M) of dione and 45 milliliters of ethanol. To this were added 7.3 grams (0.12M) of methyl vinyl ketone in two milliliters of ethanol. The reaction was charged with 0.07 milliliters of concentrated sulfuric acid and allowed to come to reflux temperature. After refluxing for 15 hours, it was cooled to room temperature and extracted with NaHCO<sub>3</sub> solution. It was subsequently extracted three times with water. The water layer was twice extracted with ether and chloroform, respectively. The organic layers were then combined and dried over CaCl<sub>2</sub>. They were subsequently flash evaporated. The remaining residue was column chromatographed. Most of the mixture consisted of unreacted dione; however, a small amount of a white crystalline material was collected and subjected to mass spectral analysis. A parent peak was seen at 228 mass units, indicating the presence of the annulated dione. Also present was the retro-Diels-Alder product at 162 and 66 mass units. The yield of this reaction was less than 0.001%.

Attempted synthesis of endo-tricyclo[6.2.1.0<sup>2,7</sup>]  
undec-9-en-3-cyanocarboxymethylidene-6-ol

Attempt 1. Into a 100 milliliter round bottom flask were placed 20 milliliters of ethanol. After the ethanol had been lowered to ice temperature, five grams (0.026M) of dione, 2.94 grams (0.036M) of ethyl cyanoacetate, and 20 drops of piperidine were added.<sup>26</sup> The reaction mixture stirred for four hours. At the end of this time, the solution was extracted with ammonium chloride solution and the organic layer, dried over Na<sub>2</sub>SO<sub>4</sub>. Subsequently, it was flash evaporated. Spectral analysis of the residue showed the reaction had failed.

Attempt 2. All conditions were the same as in attempt 1, except that the reaction was done at room temperature. The reaction again failed.

Attempt 3. All conditions were the same as in attempt 1, except that a nitrogen atmosphere was maintained, one equivalent of sodium ethoxide<sup>27</sup> was substituted for piperidine, and the reaction was conducted at room temperature. This reaction also failed, and the dione appeared to have retrograded.



Attempted preparation of the ethylene glycol ketal of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3, 6-dione

Attempt 1. Into a 300 milliliter round bottom flask fitted with a West condenser on a Dean-Stark apparatus were poured 100 milliliters of benzene. To this solution were added 10 grams (0.06M) of the dione, 17.6 grams (0.28M) of ethylene glycol, and 0.5 grams of p-toluenesulfonic acid.<sup>21</sup> The reaction mixture was refluxed until all of the water had azeotroped. The benzene solution was then cooled and extracted three times with Na<sub>2</sub>CO<sub>3</sub> solution. The organic layer was dried over CaCl<sub>2</sub> and flash evaporated. The oily residue was shown to be the mono-ketal by spectral analysis.

Attempt 2. All conditions were the same as in attempt 1, except that ten equivalents of ethylene glycol were used. This also resulted in formation of the mono-ketal.

Attempt 3. All conditions were the same as in attempt 2, except that one gram of p-toluenesulfonic acid was used. Again, only the mono-ketal was formed.

Attempt 4. All conditions were the same as in attempt 3, except that toluene was substituted for benzene. Again only a mono-ketal was formed.

Attempted carboxyethylation of the ethylene glycol mono-ketal of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3,6-dione

Attempt 1. Into a three necked, round bottom flask containing a magnetic stirring bar and fitted with a nitrogen inlet, a condenser, and a pressure equalized dropping funnel were added 2.5 grams (0.013M) of the mono-ketal in 25 milliliters of ethanol and 1.2 grams (0.013M) of diethylcarbonate.<sup>28</sup> To this mixture were added 0.013 moles of sodium ethoxide in 50 milliliters of ethanol. The reaction was refluxed under nitrogen for 24 hours. At this time, it was cooled to ice temperature and then brought to pH 7. The solution was then flash evaporated and spectral analysis of the residue showed that the reaction had failed.

Attempt 2. All conditions were the same as in attempt 1, except that the reaction was run at 0°C for 24 hours. This too failed.

Attempted synthesis of the ethylene glycol mono-ketal of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-6-cyanocarboxymethylidene-3-ol by a base condensation

Attempt 1. Into a 300 milliliter three necked, round bottom flask containing a magnetic stirring bar and fitted with a nitrogen inlet, a condenser, and a pressure equalized dropping funnel were added 100 milliliters of ethanol with 0.026 moles of sodium ethoxide.<sup>27</sup> Into this solution were inserted 2.94 grams (0.026M) of ethyl cyanoacetate. Subsequently, five grams (0.026M) of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3-ol-6-one were introduced dropwise. The reaction was brought to reflux temperature and remained there for five hours. To this point, it had been kept in a nitrogen atmosphere. Now the mixture was brought to room temperature and flash evaporated. The remaining residue was dissolved in diethyl ether and washed in a 10% solution of ammonium chloride. The ether layer was dried over Na<sub>2</sub>SO<sub>4</sub> and flash evaporated. Spectral analysis of the remaining oil showed that the reaction had failed.

Attempt 2. All conditions were the same as in attempt 1, except that in workup, the reaction was

brought to neutrality and then extracted with ethyl ether. Again the reaction failed.

Attempt 3. All conditions were the same as in attempt 2, except that the reaction was allowed to continue for 24 hours. The only thing isolated was starting material.

Attempt 4. All conditions were the same as in attempt 2, except that the temperature was kept at 0°C. This too was unsuccessful.

Attempted synthesis of the ethylene glycol ketal of endo-tricyclo 6.2.1.0<sup>2,7</sup> undec-9-en-6-cyanocarboxymethyliden-3-one by a Cope modification<sup>23</sup> of a Knoevenagel reaction

Attempt 1. Into a 300 milliliter round bottom flask containing 140 milliliters of benzene and fitted with a Dean-Stark apparatus equipped with a condenser were added 7.5 grams (0.034M) of mono-ketal of endo-tricyclo 6.2.1.0<sup>2,7</sup> undec-9-en-3,6-dione, 4.07 grams (0.036M) of ethyl cyanoacetate, and 0.28 grams (0.003M) of ammonium acetate.<sup>29</sup> The reaction was brought to reflux and continued for 24 hours. At the end of this time, all water had long since ceased to azeotrope. The mixture was cooled and extracted twice

with 100 milliliters of water. Subsequently, the organic layer was dried over  $\text{Na}_2\text{SO}_4$  and the benzene, removed by flash evaporation. Spectral analysis of the remaining crude oil showed that the reaction had not succeeded.

Attempt 2. All conditions were the same as in attempt 1, except that toluene instead of benzene was used as a solvent. This reaction was unsuccessful.

Attempt 3. All conditions were the same as in attempt 1, except that zylene instead of benzene was used as a solvent. Only starting material was isolated.

Attempt 4. All conditions were the same as in attempt 1, except that piperidine instead of ammonium acetate was used as a catalyst.<sup>26</sup> This too failed.

Attempted ethyl bromoacetate Reformansky<sup>31</sup> of the ethylene glycol mono-ketal of endo-tricyclo [6.2.1.0<sup>2,7</sup>]undec-9-en-3,6-dione

Attempt 1. Into a 300 milliliter three necked, round bottom flask containing a magnetic stirring bar and equipped with a nitrogen inlet, a Claisen head, and a pressure equalized dropping funnel were added

1.85 grams (0.03M) of freshly prepared zinc<sup>30</sup> and 200 milliliters of benzene. The benzene was distilled until approximately 100 milliliters remained. Into the dropping funnel containing 50 milliliters of benzene were added 5.0 grams (0.03M) of ethyl bromoacetate and 2.20 grams (0.01M) of the mono-ketal. From the funnel, ten drops were inserted into the round bottom. After 30 minutes at reflux temperature, no reaction was noted; whereupon, a drop of methyl magnesium was added. A reaction seemed to be initiated; therefore, the contents of the funnel were added dropwise. The reaction was allowed to reflux for an additional two hours. At this point, it was cooled to 0°C and a saturated solution of ammonium chloride was poured into the reaction vessel. The aqueous layer was separated from the organic layer and subsequently, the aqueous layer was washed three times with diethyl ether. The combined layers were dried over Na<sub>2</sub>SO<sub>4</sub> and flash evaporated. Spectral analysis of the resulting oil indicated that the reaction was unsuccessful.

Attempt 2. All conditions were the same as in attempt 1, except that a mixed solvent of diethyl ether and benzene was used.<sup>30</sup> This also failed.

Attempt 3. All conditions were the same as in attempt 1, except that toluene was used as a solvent.<sup>32</sup> This too was unsuccessful.

Attempt 4. All conditions were the same as in attempt 1, except that the ethyl bromoacetate was added before the ketone. This too failed.

Attempted annulation of the ethylene glycol mono-ketal of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3, 6-dione

Into a 300 milliliter round bottom flask fitted with a West condenser on a Dean-Stark apparatus were poured 100 milliliters of benzene. To this solution were added 5 grams (0.023M) of the mono-ketal, 1.52 grams (0.023M) of methyl vinylketone, and 0.5 grams of p-toluenesulfonic acid. The mixture was brought to reflux. At this point, the reaction turned dark brown. After five hours of azeotroping, it was cooled and flash evaporated until the volume of the solution was reduced approximately 25 milliliters. The remaining solution was dissolved in diethyl ether and gravity filtered to remove black polymeric material. The filtrate was then washed with a 5% solution of Na<sub>2</sub>CO<sub>3</sub>.

The ether was dried over  $\text{Na}_2\text{SO}_4$  and flash evaporated. The resulting oil was subjected to spectral analysis which indicated that the reaction had failed.

Attempt formylation<sup>32</sup> of the ethylene glycol mono-ketal of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3,6-dione

Into a 300 milliliter three necked, round bottom flask containing a magnetic stirring bar and fitted with a nitrogen inlet, a condenser, and a pressure equalized dropping funnel were added five grams (0.02M) of the mono-ketal in 25 milliliters of ethanol. Into the pressure equalized dropping funnel were placed 100 milliliters of ethanol and 0.52 grams (0.02M) of sodium. After the sodium had completely dissolved and the ethanol cooled to room temperature, the contents of the dropping funnel were dripped into the round bottom. Then, 1.7 grams (0.02M) of ethyl formate were added dropwise. The reaction was brought to reflux. After 9½ hours, it was cooled to ice temperature; whereupon, the pH of the solution was lowered to seven. The reaction solution was subsequently flask evaporated. Spectral analysis of the remaining brownish liquid showed that the reaction was not successful.



Attempted cyanoethylation<sup>33</sup> of the ethylene glycol mono-ketal of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3,6-dione

Attempt 1. Into a 300 milliliter three necked, round bottom flask containing a magnetic stirring bar and fitted with a nitrogen inlet, a condenser, and a pressure equalized dropping funnel were added 2.20 grams (0.01M) of the mono-ketal in 50 milliliters of ethanol. Into the pressure equalized dropping funnel were placed 50 milliliters of ethanol and 0.26 grams (0.01M) of sodium. After the sodium had completely dissolved and the ethanol cooled to room temperature, the contents of the dropping funnel were dripped into the round bottom. Then, 0.5 grams (0.01M) of acrylonitrile were added dropwise. The reaction was allowed to stir for four hours at 0°C. At this time, the pH of the solution was lowered to seven and flash evaporated. Spectral analysis of the residue indicated the reaction was not successful.

Attempt 2. All conditions were the same as in attempt 1, except that the reaction was run at reflux temperature.<sup>34</sup> This also failed.

Attempted synthesis of endo-tricyclo[6.2.1.0<sup>2,7</sup>]  
undec-9-en-3-chloro-6-one

Attempt 1. Into a three necked, round bottom flask containing 120 milliliters of benzene and fitted with a condenser and pressure equalized dropping funnel were added 5.0 grams (0.026M) of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3-ol-6-one and one milliliter of pyridine. Next, 3.2 grams (0.026M) of thionyl chloride were inserted dropwise over a 30 minute period.<sup>35</sup> At the end of this time, the reaction mixture was brought to reflux temperature. After six hours, the reaction was cooled and extracted with water, Na<sub>2</sub>CO<sub>3</sub> solution, and brine water. The organic layer was collected and dried over Na<sub>2</sub>SO<sub>4</sub>, and subsequently flash evaporated. Spectral analysis of the residue showed that the reaction was unsuccessful.

Attempt 2. All conditions were the same as in attempt 1, except that phosphorous trichloride was substituted for thionyl chloride.<sup>36</sup> The reaction failed.

Attempt 3. Into 100 milliliters of dried carbontetrachloride were added 5.0 grams (0.026M) of the keto-alcohol and 6.7 grams (0.026M) of triphenyl

phosphine.<sup>37</sup> The reaction mixture was brought to reflux temperature for six hours; whereupon, it was cooled to room temperature and the organic layer, extracted three times with ammonium chloride solution. the carbontetrachloride was then reduced in volume by flask evaporation and subjected to spectral analysis. There was no trace of the starting keto-alcohol. The water layer was then re-extracted with ether, and the ether layer was flash evaporated. There was still no trace of the keto-alcohol. The entire reaction was repeated with the same results. Apparently, the triphenylphosphine initiates a retro-Diels-Alder.

Attempt 4.<sup>38</sup> Into 100 milliliters of dry tetrahydrofuran were added 2.2 grams (0.01M) of the monoketal alcohol. To the solution were added 15 milliliters of 2M methyllithium in solvent. After the ebullition of hydrogen had ceased, the reaction was allowed to stir for an additional 30 minutes. At this time, 1.5 grams (0.013M) of methyl sulfonylchloride were added dropwise. The reaction mixture stirred for an additional hour, whereupon 3.4 grams (0.04M) of lithium bromide were inserted. The reaction stirred for an additional hour; then it was washed with an ammonium chloride solution. The organic layer was collected and dried over  $\text{MgSO}_4$  and subsequently flash evaporated.

Spectral analysis indicated that the reaction failed.

Attempted formation of the oxime of  
endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3-ol-6-one

Into a 125 milliliter Erlenmeyer flask were placed 50 milliliters of dimethylsulfoxide, five grams (0.026M) of the keto-alcohol, and 0.9 grams (0.026M) of hydroxylamine. The Erlenmeyer flask was put into a heating bath of approximately 90°C for three hours. The solution was then poured into 200 milliliters of water and extracted with diethyl ether. The diethyl ether was then flash evaporated and the remaining residue was subjected to spectral analysis. The reaction failed.

Attempted trapping of the cyanoethylation of  
endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3-ol-6-one

Into a 300 milliliter three necked, round bottom flask containing a magnetic stirring bar and fitted with a nitrogen inlet, a condenser, and a

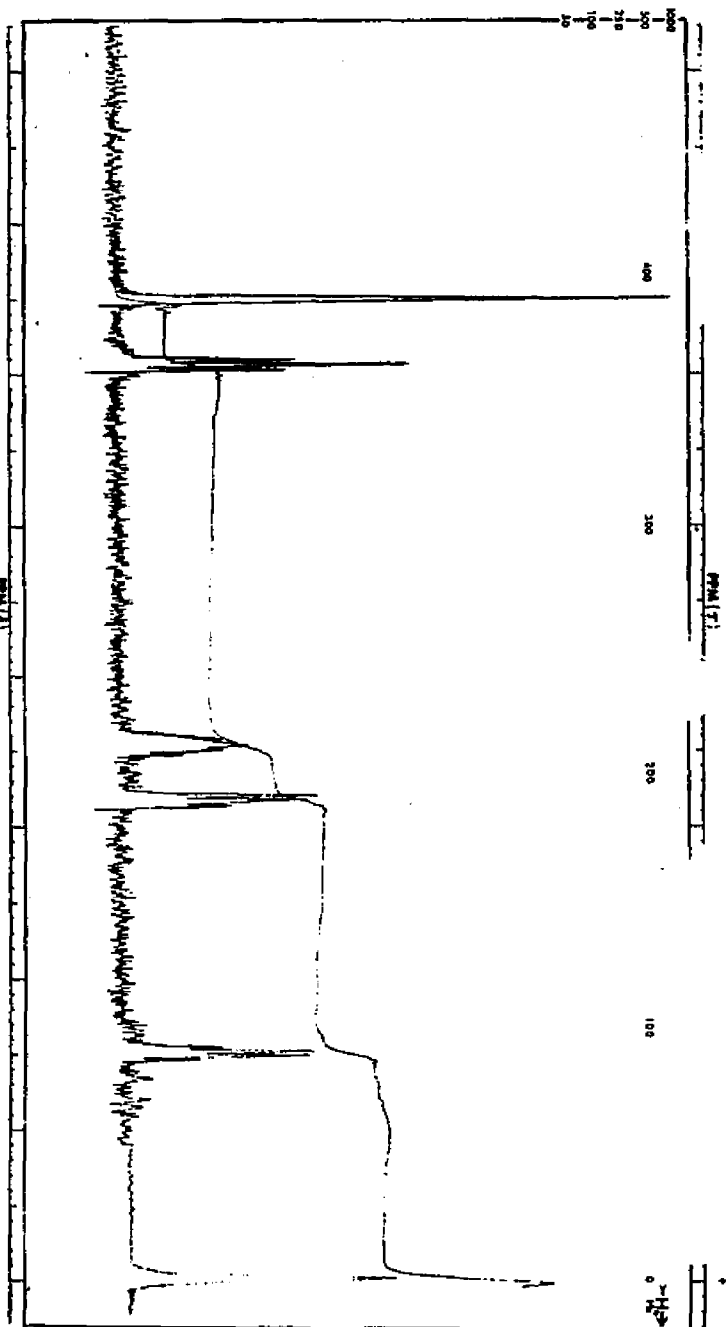
pressure equalized dropping funnel were added 7.1 grams (0.04M) of the mono-reduced ketone in 50 milliliters of ethanol. Into the pressure equalized dropping funnel were placed 50 milliliters of ethanol and 1.0 grams (0.04M) of sodium. After the sodium had completely dissolved and the ethanol cooled to room temperature, the contents of the dropping funnel were dripped into the round bottom. The 2.0 grams (0.04M) of acrylonitrile were added dropwise. The reaction stirred for eight hours at 0°C. Then, 7.4 grams (0.02M) of sodium borohydride were inserted into the reaction mixture. After three hours, 20 milliliters of water were added and the contents of the round bottom, poured into an Erlenmeyer flask. The volume of the solution in the Erlenmeyer flask was reduced to approximately 30 milliliters by boiling. After the solution had cooled, it was extracted with diethyl ether and the ether layer was then flash evaporated. The remaining oil was crystallized in chloroform. Spectral analysis seemed to indicate the presence of the diol. A mixed melting point of this crystal with an authentic sample of the diol showed no melting point depression.

Attempted synthesis of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3-ol-6-cyanocarboxymethylidene

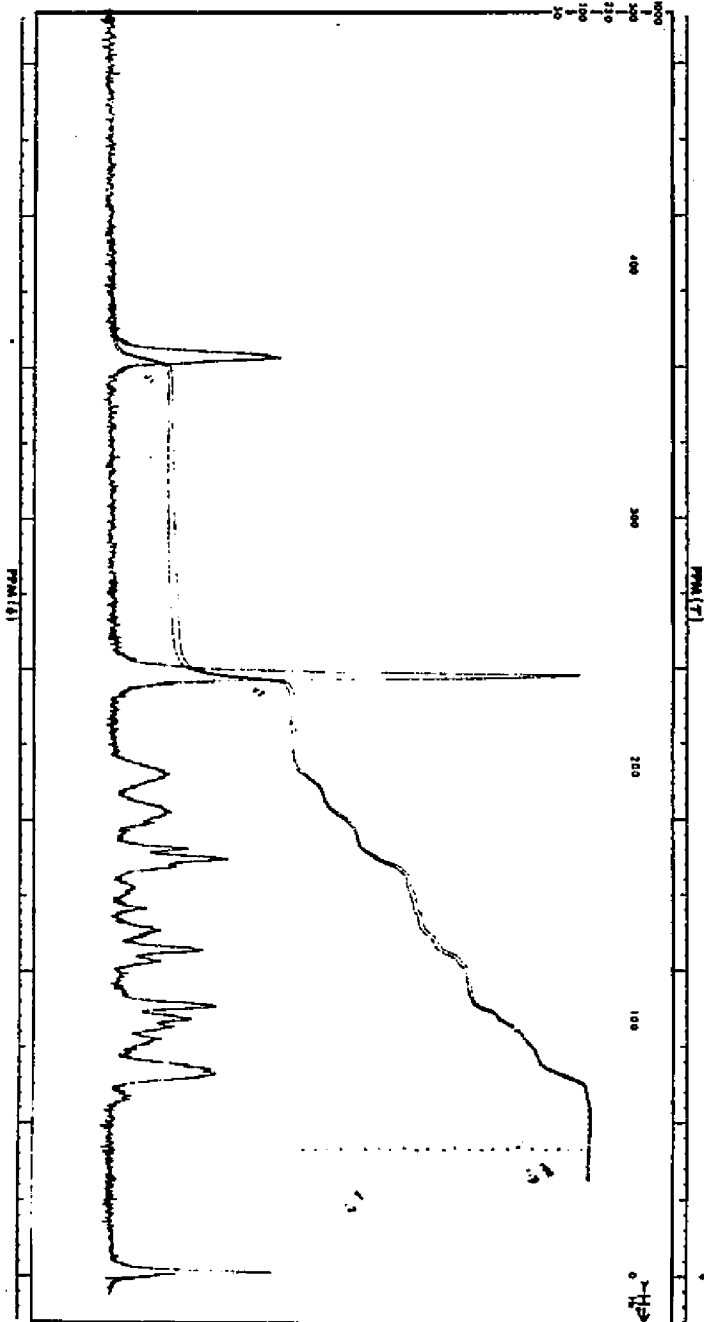
Attempt 1. Into a 300 milliliter round bottom flask containing 120 milliliters of benzene and fitted with a Dean-Stark apparatus equipped with a condenser were added 5.0 grams (0.026M) of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3-ol-6-one, 3.0 grams (0.0265M) of ethyl cyanoacetate, and 0.2 grams (0.0026M) of ammonium acetate.<sup>29</sup> The reaction was brought to reflux and continued for 15 hours. At the end of this time, all water had long since azeotroped. The mixture was cooled and extracted twice with 100 milliliters of water. Subsequently, the organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> and the benzene removed by flash evaporated. Spectral analysis of the remaining crude oil showed that the reaction had failed.

Attempt 2. All conditions were the same as in attempt 1, except that toluene instead of benzene was used as a solvent. This too was unsuccessful.

Attempt 3. All conditions were the same as in attempt 1, except that piperidine<sup>26</sup> instead of ammonium acetate was used as a catalyst. It failed.

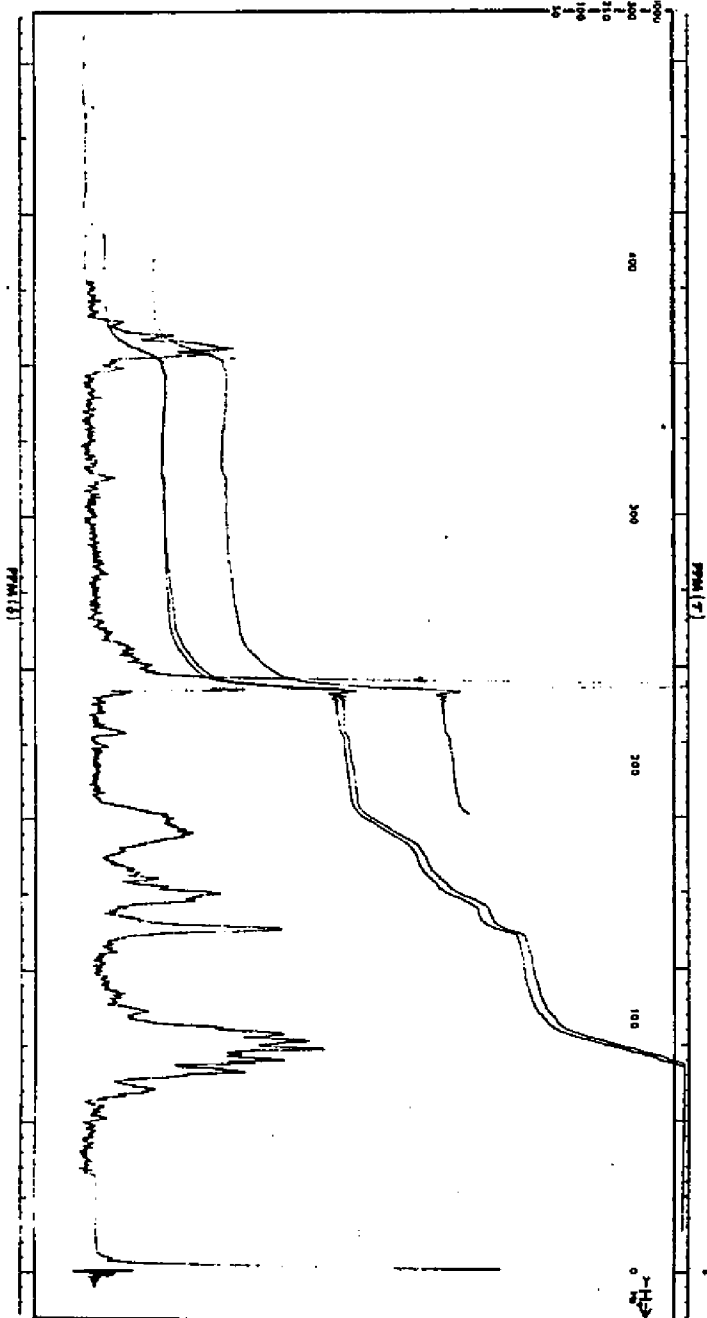


endo-tricyclo[6.2.1.0<sup>3,7</sup>]undec-4,9-dien-3,6-dione

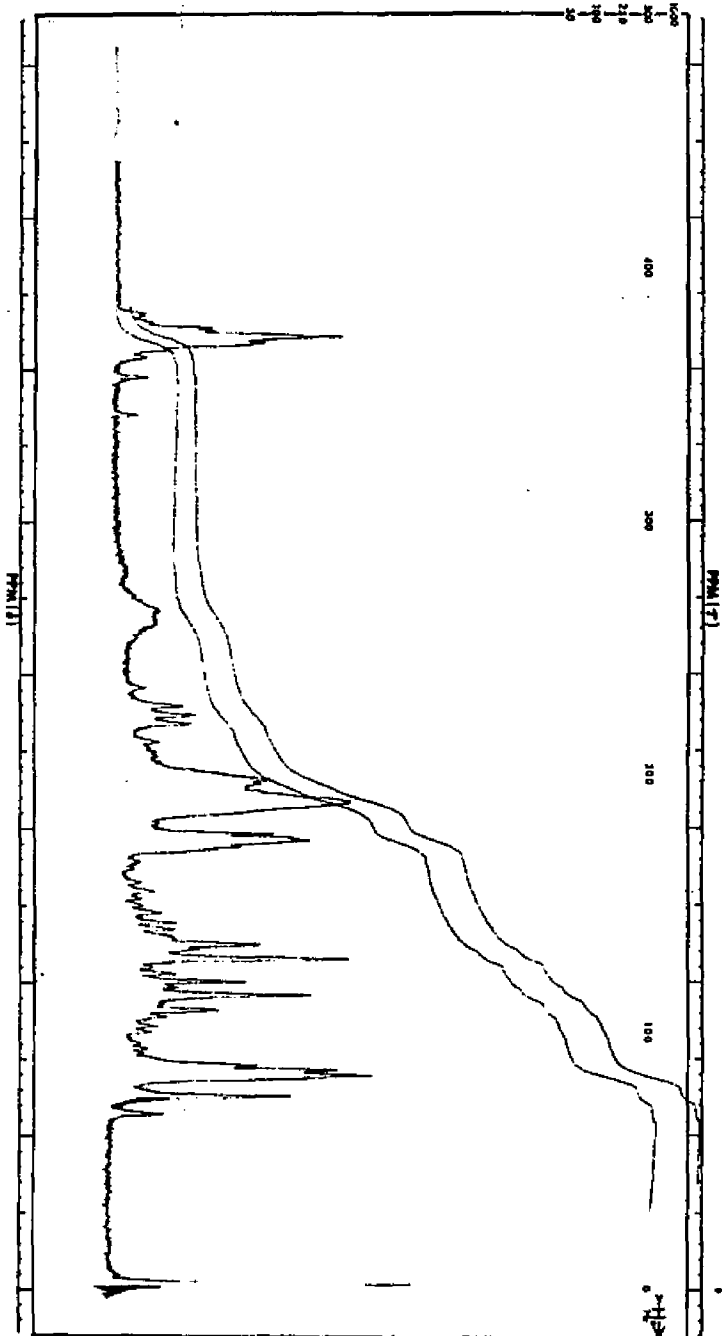


ethylene glycol mono-ketal of endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3,6-diene

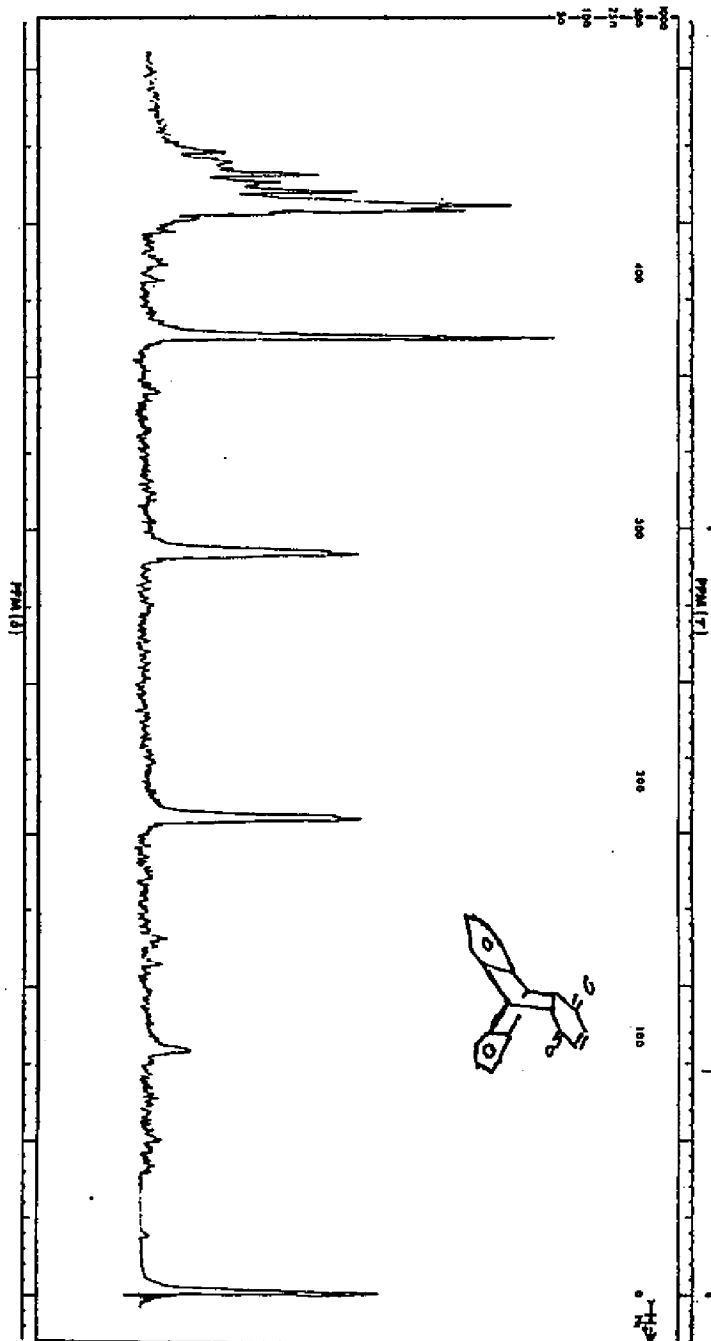


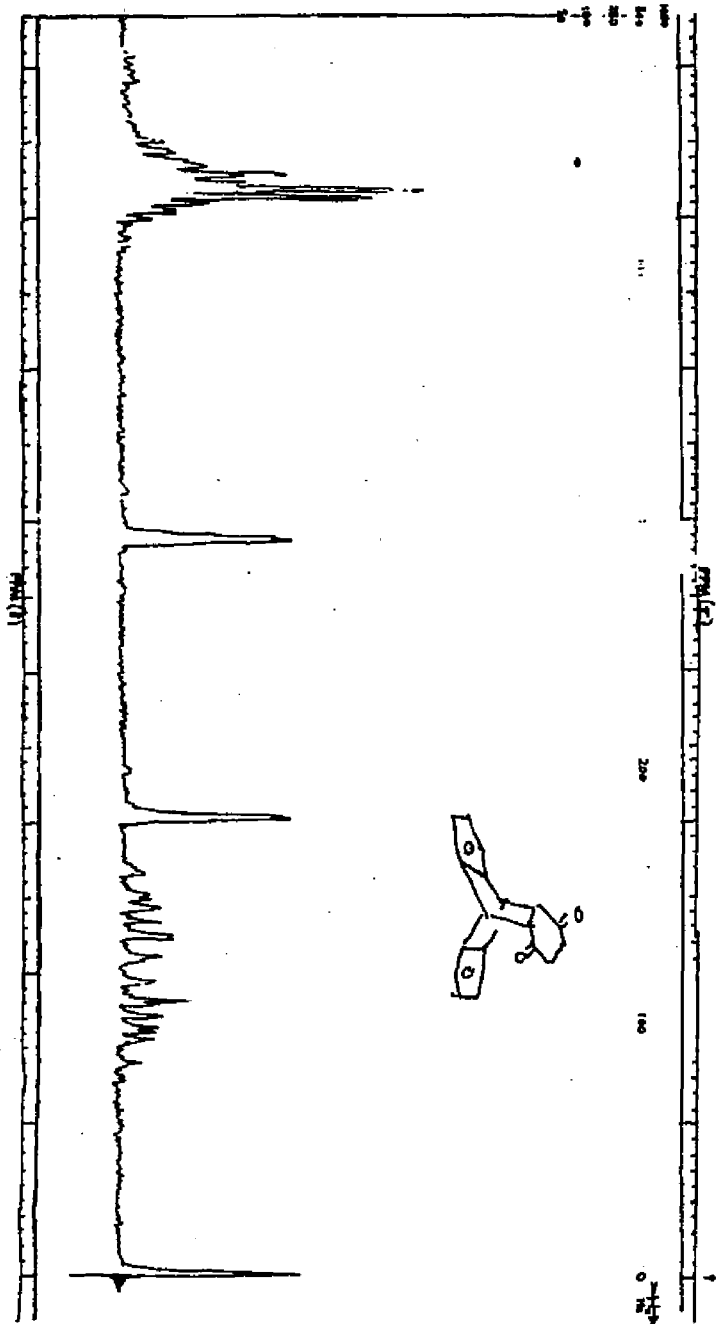


ethylene glycol of endo-tricyclo[6.2.1.0<sup>3,7</sup>]undec-9-en-3-ol-6-one



endo-tricyclo[6.2.1.0<sup>2,7</sup>]undec-9-en-3-ol-6-one





APPENDIX  
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## VITA

Angelo Russo, born on April 9, 1946, in New Orleans, Louisiana, received his Bachelor of Science degree from Louisiana State University in Baton Rouge in 1969, and is presently a candidate for the Doctorate in Philosophy at Louisiana State University. He is married to Mary Lee Hirt Russo and is the father of two boys, Hud and Dylan.



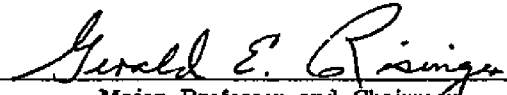
# EXAMINATION AND THESIS REPORT

Candidate: Angelo Russo

Major Field: Biochemistry


Title of Thesis: STUDIES DIRECTED TOWARD THE ISOLATION AND STRUCTURE DETERMINATION  
OF SEX AND FOOD ATTRACTANT OF CYLAS FORMICARIUS ELEGANTULUS (SUM.)

Approved:

  
Major Professor and Chairman

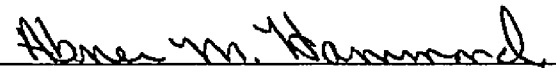
  
Dean of the Graduate School

## EXAMINING COMMITTEE:









Date of Examination:

September 4, 1973